experimentation is undue, an analysis of the factors set forth in *In re Wands* is required. *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). The Examiner has analyzed each of these factors, but Applicants respectfully maintain that the arguments presented do not support the Examiner's conclusion that experimentation is undue. Each of these factors is addressed below.

Nature of the Invention: The claims are drawn to methods for treating a subject having or at risk of developing a cancer using an immunostimulatory nucleic acid in combination with a cancer medicament to treat or reduce the risk of developing the cancer. In some aspects, the nucleic acid is not conjugated to the cancer medicament. The invention is within the field of cancer therapy, and can be characterized as an immunotherapy to the extent that the immunostimulatory nucleic acid is immunostimulatory (i.e., it is able to stimulate innate and/or adaptive immune responses upon administration). Thus the nucleic acid is able to stimulate and/or enhance an immune response directed at a cancer.

Breadth of the Claims: The pending claims encompass treating or preventing a cancer in a subject having or at risk of developing cancer by administering a poly-G nucleic acid in conjunction with a cancer medicament in an effective amount. The pending claims also encompass treating or preventing a cancer in a subject having or at risk of developing a cancer by administering an immunostimulatory nucleic acid in conjunction with a cancer medicament that is a hormone therapy. The specification defines a poly-G nucleic acid starting on page 12, line 13 and provides examples of poly-G nucleic acids in SEQ ID NO: 95 – SEQ ID NO: 133 as well as SEQ ID NOs: 46, 47, 58 and 61. Moreover, as evidenced by the cited references on page 12, the art is familiar with poly-G nucleic acids. The specification further defines CpG nucleic acids starting on page 10, line 13 and provides examples of CpG nucleic acids in published PCT applications PCT/US95/01570 and PCT/US97/19791. Non-CpG immunostimulatory nucleic acids are also defined starting on page 12, line 7 and examples include poly-G nucleic acids (as described above) and T-rich nucleic acids (see page 13, lines 1-2). Further examples of T-rich nucleic acids are provided in published PCT application PCT/US00/26383.

Additionally, cancer is a recognized disease, and the art is familiar with its diagnosis and in some cases treatment (even if only partial). The subject to be treated according to the invention can be any subject that has or is at risk of developing a cancer. Human and non-human subjects (e.g., dogs, cats, livestock) are known to develop cancers. The teaching of the

specification and the state of the art at the time of filing are commensurate with the breadth of the pending claims.

Guidance Provided: The specification provides sufficient guidance to enable one of ordinary skill in the art to make immunostimulatory nucleic acids. Examples of immunostimulatory nucleic acids (CpG or non-CpG-containing nucleic acids, such as poly-G nucleic acids) are provided through the general formulae on page 10, lines 26-32; page 11, lines 1-15 and page 12, lines 23-28. In addition, an extensive list of exemplary immunostimulatory nucleic acid sequences is given on pages 12 through 15. One of ordinary skill in the art would merely use routine methods to make the immunostimulatory nucleic acids of the present invention.

The specification also provides sufficient guidance to enable one of ordinary skill in the art to use the immunostimulatory nucleic acids, specifically in combination with a cancer medicament. The immune effects of immunostimulatory nucleic acids have been extensively described in US Patent No. 6,194,388 and in the published patent applications listed on page 10, lines 10 and 11. References which describe the immunostimulatory properties of poly-G nucleic acids are provided in the specification on page 12, lines 14-19. Murine tumor models, which are known in the art, have been used to show that CpG and poly-G nucleic acids have anti-tumor effects. CpG immunostimulatory nucleic acids have been found to be effective in at least the following cancers (as evidenced by murine or rodent models): leukemia, lymphoma, melanoma, neuroblastoma, cervical cancer, prostate cancer Lewis lung cancer, renal cell cancer and glioma. For instance, Figures 1-4 of Appendix B show a decrease in tumor volume and an increase in survival due to the administration of CpG nucleic acids in neuroblastoma and cervical cancer mouse models. The anti-tumor effects of CpG nucleic acids are also illustrated in Figure 5 of Appendix B, where CpG nucleic acids were found to increase the survival of mice injected with murine B16 melanoma cells. Moreover, the anti-cancer effects of CpG monotherapy in a murine T cell lymphoma model are illustrated in Figure 6 of Appendix B. Shen et al. reported that CpG and poly-G nucleic acids, when used as a monotherapy, decreased tumor volume (with and without tumor resection) and increased survival in mice (Shen, et al., 2002, Appendix A). Similarly, Blazar et al. reported increased survival in mice when CpG nucleic acids were administered prior to acute myelogenous leukemia challenge (Appendix C). Anti-tumor effects of poly-G nucleic acids have been observed in a prostate cancer murine model.

Cancer medicaments and their use in cancer therapy are known in the art. Detailed lists of a variety of cancer medicaments are given in Tables 4, 5 and 6 of the specification. These therapeutics include immunotherapies, vaccines and chemotherapies. In order to use the combinations of the claimed invention, one of ordinary skill in the art assays for the desired immune response and determines the effective amount using methods known in the art and provided in the specification.

The Examiner concedes that the specification discloses a variety of protocols for administering the compositions; however, the Examiner also states that no guidance is provided to instruct one of ordinary skill as to the dosage amount or frequency required to treat a subject with cancer. Applicants respectfully disagree. The specification provides adequate guidance regarding therapeutic dosages, as well as methods for assessing the efficacy of the combinations of the claimed invention. For example, Applicants have described the route, timing and frequency of administration on pages 50 and 51. Effective amounts are described on page 20, lines 3-16 and page 47, lines 25-31 lines. Methods for determining effective amounts are provided throughout pages 49 and 50. These include *in vitro* or *in vivo* cytokine production assays, cell culture assays, *in vitro* stimulation assays, in animal studies and human clinical trials, etc. Those of ordinary skill will be able to determine the exact dose to administer without undue experimentation based on the level of ordinary skill in the art and the teachings of the specification.

Applicants teach on page 7, line 33 through page 8, line 1 of the specification that immunostimulatory nucleic acids can be used in combination with cancer medicaments to treat cancer more effectively by way of modulating the immune response. The invention is premised in part on the ability of immunostimulatory nucleic acids such as CpG and poly-G nucleic acids to induce an immune response *in vivo*. CpG and poly-G nucleic acids induce Th1 immune responses when administered *in vivo*. Both nucleic acid types exhibit anti-tumor effects when administered *in vivo* (as described herein and in Shen, et al., 2002, Appendix A). Combinations of CpG nucleic acids with cancer medicaments are more effective than either agent alone in a number murine cancer models. The combination of Taxol® and CpG increased survival and decreased tumor volume in a murine model of Lewis lung cancer (Appendix B, Figures 7-8). CpG in combination with Xeloda® decreased tumor volume in a murine model of renal cell cancer (Appendix B, Figure 9). Combinations of CpG nucleic acids with the cancer medicament cyclophosphamide has also been shown to prolong survival in a mouse rhabdomyosarcoma

model (Appendix B, Figures 10-13). Briefly, mice were injected with tumor cells at day 0. One group of mice with sub-clinical tumors at day 9 were administered cyclophosphoramide in combination with CpG nucleic acids. A second group of mice with clinical tumors were administered the same combination at Day 19. The data presented in Figures 10-13 illustrate the effectiveness of this combination. The anti-cancer effects are evidenced by improved survival and decreased tumor volume in the mice. Accordingly, since CpG nucleic acids are able to potentiate the anti-tumor effects of chemotherapeutic agents (e.g., cyclophosphamide), as demonstrated by the data in Appendix A, then so to can poly-G nucleic acids. In view of the foregoing, one of skill in the art would have no reason to doubt that a correlation exists between the therapeutic combinations of immunostimulatory nucleic acids and cancer medicaments taught by Applicants and the claimed invention of treating or preventing cancer in a subject.

If the Examiner prefers, Applicants can submit a declaration to support the data of Appendix A. Applicants remind the Examiner that the post-filing references cited herein, as well as the data presented in Appendix A, serve only to provide evidence that the specification was enabling at the time of filing. Applicants do not rely on these references or data to enable the claimed invention.

State of the Art: The state of the art at the time of filing the instant application is such that one of ordinary skill knew *inter alia* of the anti-cancer effects of immunostimulatory nucleic acids. Specifically, U.S. patent 6,239,116 and published PCT application PCT/EP99/06502, teach the benefits of using immunostimulatory nucleic acids for the treatment of cancer. Yaswen et al., 1993, reported that with the administration of poly-G nucleic acids *in vitro* growth of immortalized human epithelial cells was diminished (Appendix D). Published PCT application PCT/US97/19791 describes the use of poly-G nucleic acids in cancer treatment. The state of the art at the time of filing also allowed one of ordinary skill in the art to make, test and use the immunostimulatory nucleic acids of the claimed invention.

Level of Ordinary Skill: Applicants agree with the Examiner's assessment that the level of skill in the art is high, and therefore, Applicants again maintain that one of skill in the art is enabled to make and use the immunostimulatory molecules according to the claimed methods. The level of skill in the art has an important effect on the amount of guidance which must be provided to enable the invention. As the court stated in *In re Howarth*, "[i]n exchange for the patent, [the applicant] must enable others to practice his invention. An inventor need not, however, explain every detail since he is speaking to those skilled in the art." *In re Howarth*,

654 F.2d 103, 105 (CCPA 1981). Moreover, one of ordinary skill in the art is accustomed to complex experimentation, and thus the experimentation required to make and use the claimed invention would be considered routine to the ordinary artisan. Based on the high level of skill and the complex experimentation that is routinely performed in the art, one of ordinary skill would be able to make, test and use the immunostimulatory nucleic acids and, therefore, be able to practice the claimed invention without undue experimentation.

Predictability of the Art: The Examiner maintains that the specification does not provide guidance in order to overcome the unpredictability of cancer therapy. As evidence of such unpredictability the Examiner cites two references. The first reference is an article by Gouttefangeas et. al., 2000 which teaches that immunotherapy has remained elusive because "...no or not enough cancer antigens are known...(and) no consensus exists for the best antigen formulation or the route of immunization...(and) tumors under immune attack tend to be selected antigen loss variants". The kind of immunotherapy addressed in Gouttefangeas et al. is, however, unlike the immunotherapy of the claimed invention. The methods of Gouttefangeas et al. require the identification of cancer antigens and their formulation and administration *in vivo*. The claimed invention is not so dependent. Rather, in most aspects, the claimed invention takes advantage of tumor lysis *in vivo* (via the administered cancer medicaments and also the immunostimulation nucleic acids) to expose and/or release antigens from the targeted tumor. The administered nucleic acids heighten the immune system so that when activated immune cells come in contact with tumor debris, they will more effectively mount responses to it. Accordingly, in some aspects, there is no need for exogenous antigen administration as the cancer or tumor antigens are provided endogenously. Gouttefangeas et al. speaks to the unpredictability of using exogenous cancer antigens to stimulate an immune response *in vivo*, while Applicants' invention is the use of immunostimulatory nucleic acids in combination with a cancer medicament. None of the teachings in Gouttefangeas et al. are relevant to the predictable nature of immunostimulatory nucleic acids in the treatment of cancer alone or in combination with cancer medicaments. Interestingly, the teachings of Gouttefangeas et al. regarding the use of CpG immunostimulatory sequences actually support the use of these sequences as adjuvants in the vaccination of mice with antigen. The article also details the use of CpG sequences coupled covalently to ovalbumin in mice, resulting in the successful induction of CTL activity against cells expressing the protein. This evidence bolsters the arguments presented herein regarding the use of immunostimulatory nucleic acids in the claimed invention.

The Examiner further argues that Gouttefangeas et al. teaches that the effects in humans have yet to be demonstrated and the "...the efficacy of CpG-protein constructs for immunotherapy in patients remains to be tested." The claimed methods are not dependent upon and in some cases exclude conjugates of nucleic acids and antigens. Notwithstanding this, however, human testing is the mandate of the FDA, not the Patent Office. Patentability is not dependent on a showing of efficacy in a human subject. The data presented indicate the nucleic acids in combination with cancer medicaments have anti-tumor effects that are greater than the effects of either agent alone. The burden is on the Examiner to indicate why such data is not correlative to the claimed methods.

The Examiner also cites Old, 1996 for the premise that immunotherapy is unpredictable. The instant application was filed on March 5, 2001, with a priority date of March 3, 2000. The Old reference cited by the Examiner dates back to 1996, three and a half years before the earliest priority date of the instant application. Accordingly, the relevance of this article to the "state of the art at the time of filing" is tenuous at best.

Regardless, Old states that cancer cells effectively evade immune recognition by altering themselves in order to avoid attack. Old further points out that cancer vaccines may injure normal cells. The Examiner concludes that there are obstacles to overcome for immunotherapy, and the specification has not offered "direct, correlative guidance on how to administer the therapeutic composition" in order to "consistently, effectively, and predictably" treat every type of cancer.

Applicants again respectfully disagree with the Examiner's assessment. Old's teachings address immunotherapies very generally and broadly. Particular immunotherapies using antibodies, antigens or adoptive immunity have little bearing on the use of immunostimulatory nucleic acids in combination with a cancer medicament for the reasons stated above. The methods of Old require an *a priori* knowledge of cancer antigens. The claimed methods in most aspects do not. Moreover, the relevance of immune evasion of cancer cells is questionable given that the claimed methods provide for a continual attack on tumors *in vivo* without reliance on one or a defined subset of tumor antigens. According to the claimed invention, the immune system will maintain a response to whatever antigens are presented by the tumor or which are exposed and/or released following tumor lysis by the cancer medicament. The improvement provided by the immunotherapy in combination with the cancer medicament can also be the result of, for example, the stimulation of hematopoietic recovery, anti-microbial infection activity,

enhancement of uptake of the cancer medicament, inhibition or prevention of allergic responses, etc. as taught in the specification. Old's teachings do not specifically address the use of immunostimulatory nucleic acids in such combinations. The teachings of Old do not negate the use of immunostimulatory nucleic acids with known cancer medicaments.

The Examiner states that it would be trial and error to make and use the therapeutic molecules of the claims. Applicants reiterate that in view of the teachings of the specification and knowledge in the art, one of ordinary skill (which the Examiner acknowledges is high) is able to practice the claimed methods without undue experimentation.

Working Examples: The Examiner points out that no working examples of effective treatment of any cancer are provided in the specification. Applicants maintain that an absence of working examples is not dispositive to the enablement of the claimed methods. A working example need not be provided if the invention is otherwise disclosed in a manner such that one of ordinary skill would be able to practice the invention without undue experimentation. Applicants maintain that, with the guidance provided as well as the level of skill in the art, this is the case.

Amount of Experimentation: The Examiner also argues that the quantity of experimentation is very large and would require the use of animal testing and, ultimately, human testing. The Applicants, however, maintain that the experimentation required to make and use the claimed invention is not great and is commensurate with that which the art routinely engages in. It is considered routine in the art to engage in animal testing and subsequent human testing to evaluate therapeutics. For this level of experimentation to be undue, it must be demonstrated that the experimentation is more than typically engaged in in the art. Requiring complex or even a large amount of experimentation is not sufficient to make it undue, if the art routinely engages in this level of experimentation. Applicants assert that this level of experimentation is typically engaged in in the art, and accordingly this kind of experimentation is routine and not undue.

In view of the foregoing, one of ordinary skill in the art would engage in no more than routine experimentation to practice the claimed invention. Applicants respectfully request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. §112, first paragraph.

Summary

It is believed that all of the pending claims are in condition for allowance. If the Examiner has any questions or comments, he is encouraged to contact Applicants' representative at the number listed below.

Respectfully Submitted,

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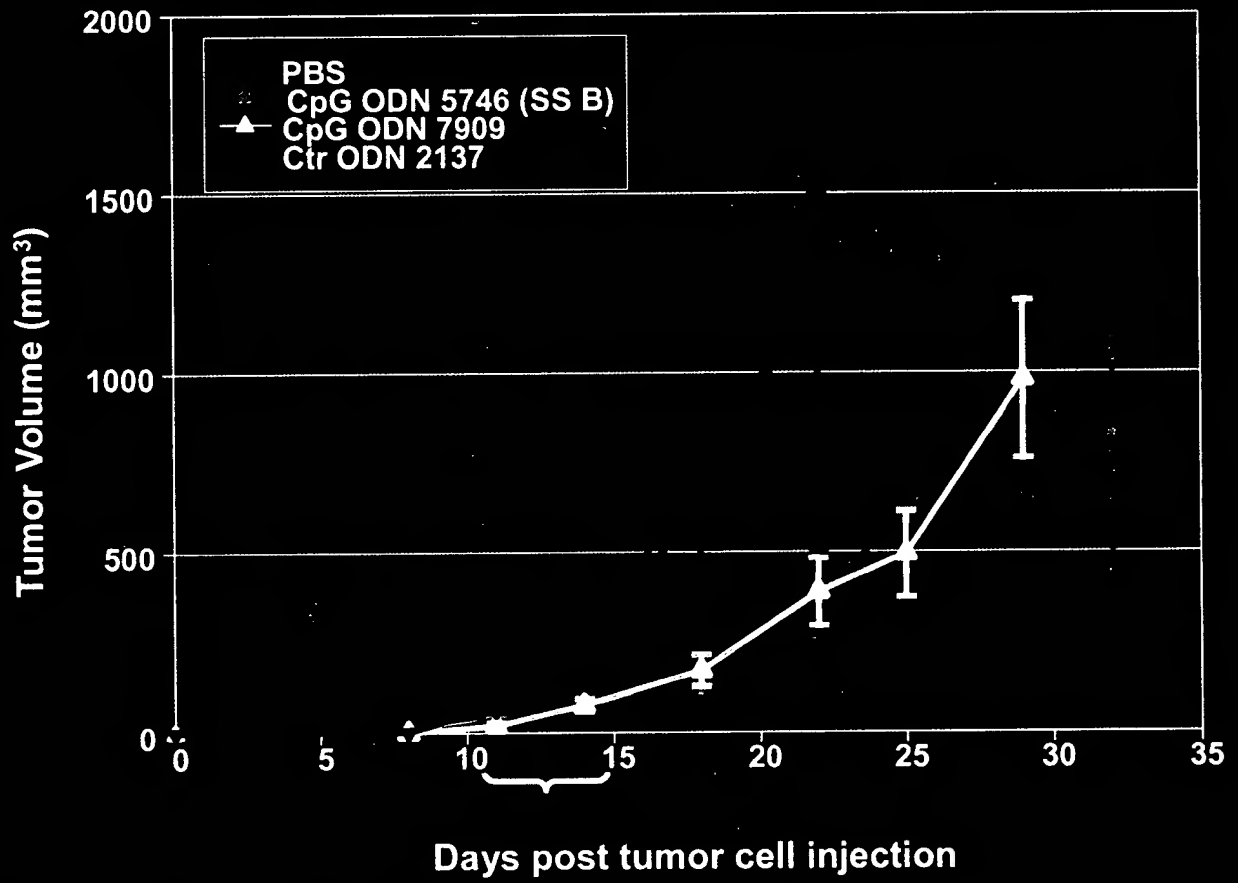
Docket No.: C01037.70017.US
Date: February 28, 2003
X02.28.03

MURINE NEUROBLASTOMA MODEL: CpG MONOTHERAPY W/O TUMOR RESECTION



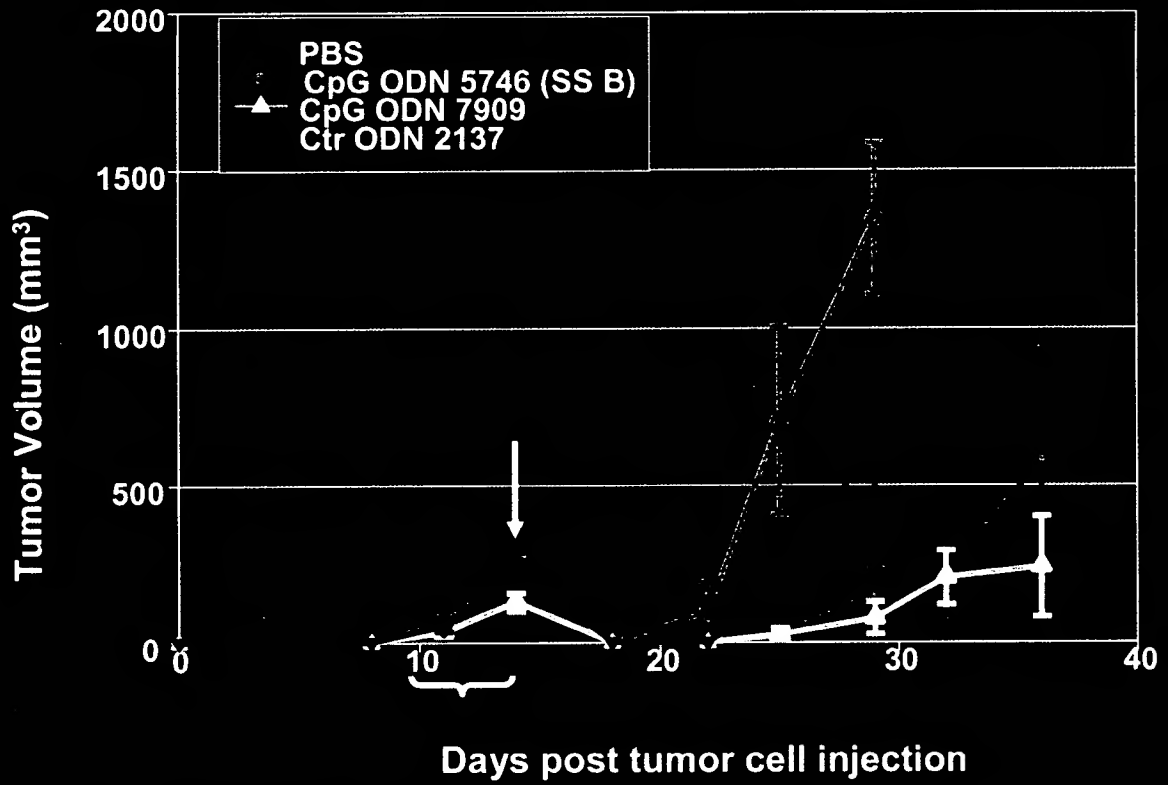
Tumor induction: 1x10^6 Neuro2a cells injected SC in the left flank on Day 0

Treatment: SC injections of PBS or 100µg ODN 5746, 7909 or 2137 daily from Day 10 to Day 15.

FIGURE 1

APPENDIX B

MURINE NEUROBLASTOMA MODEL: CpG MONOTHERAPY WITH TUMOR RESECTION



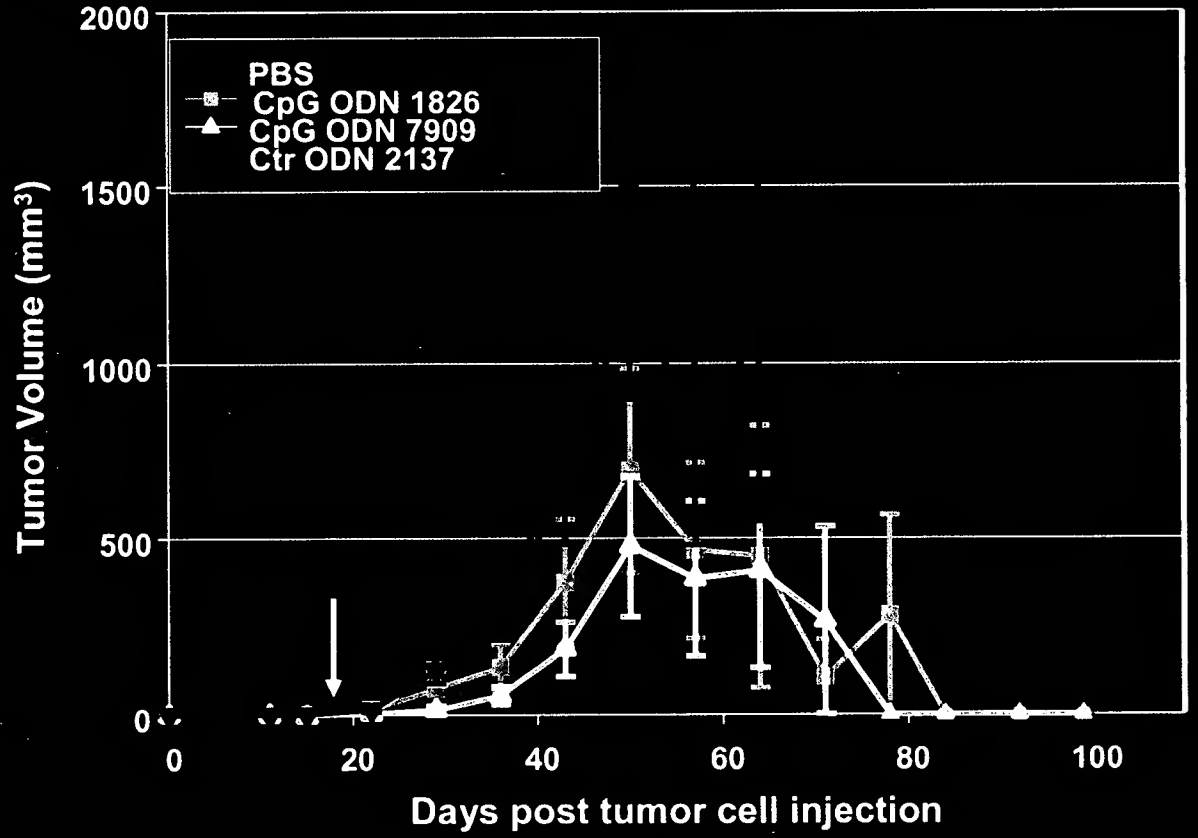
Tumor induction: $1x10^6$ Neuro2a cells injected SC in the left flank on Day 0

Tumor Removal: Tumors were surgically removed on Day 15; wound washed with PBS or 100µg of ODN 5746, 7909 or 2137

Treatment: SC injections of PBS or 100µg ODN 5746, 7909 or 2137 daily from Day 10 to Day 15.

FIGURE 2

MURINE CERVICAL CARCINOMA MODEL: CpG MONOTHERAPY

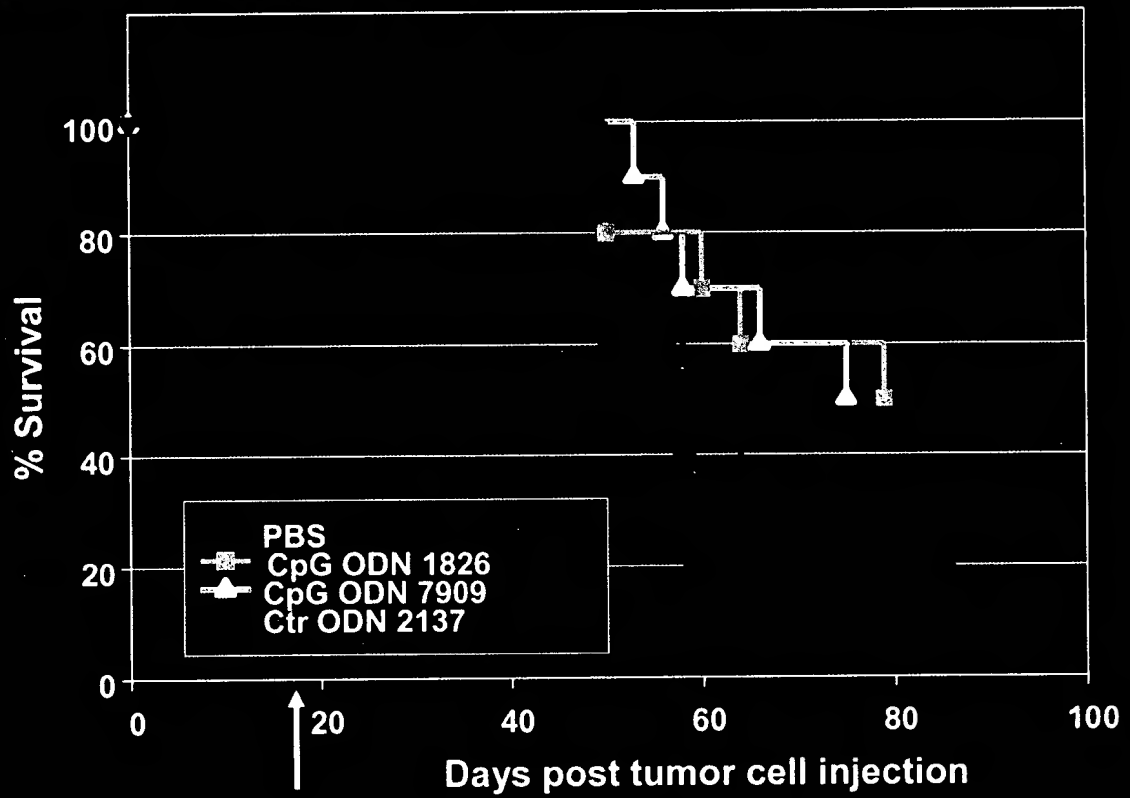


Tumor induction: 1x10^4 cervical carcinoma cells injected SC in the left flank on Day 0

Treatment: SC injections (nape of neck) of PBS or 150μg ODN 7909, 1826 or 2137 daily from Day 10 to Day 18.

FIGURE 3

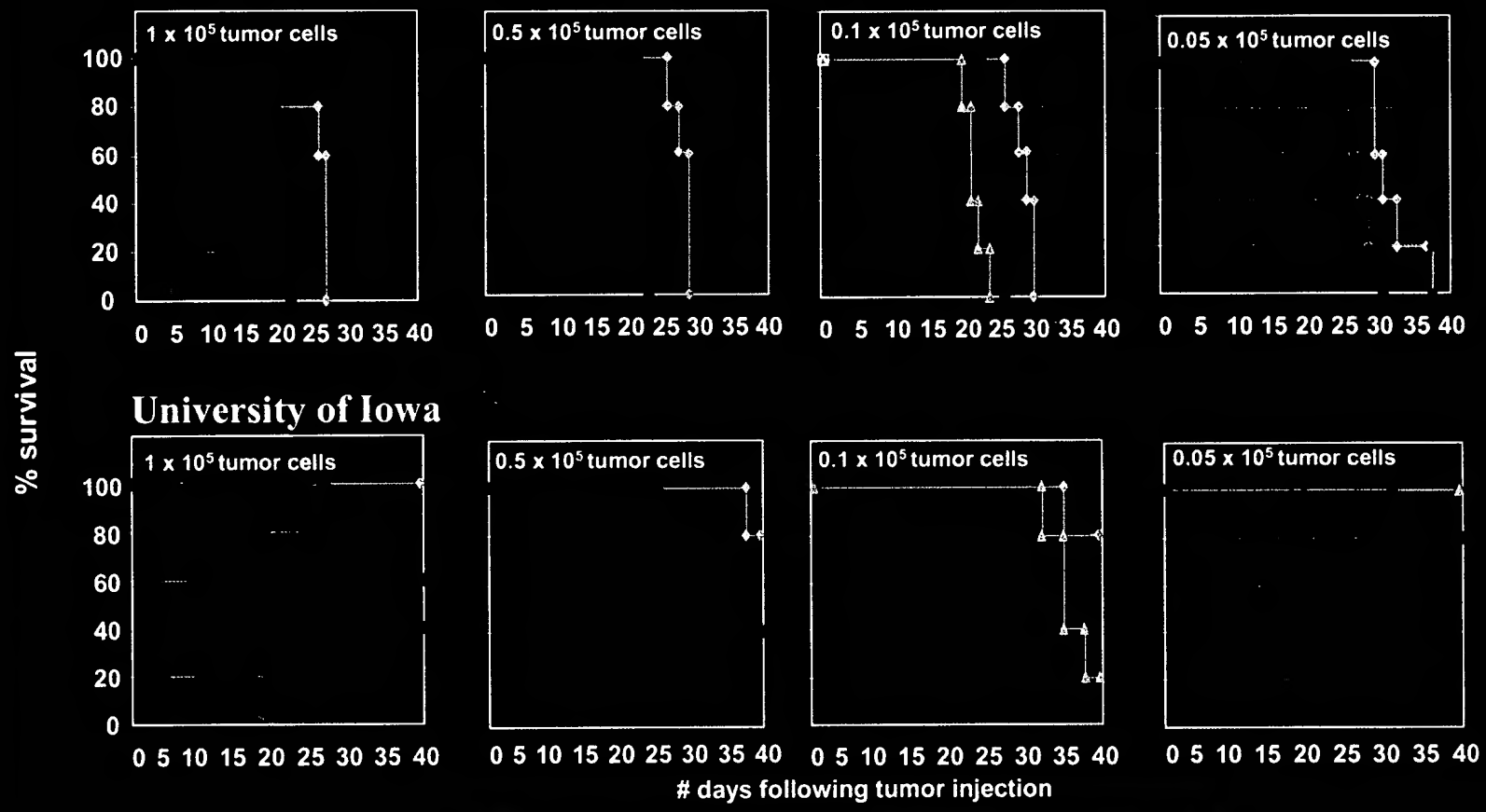
MURINE CERVICAL CARCINOMA MODEL: CpG MONOTHERAPY



Tumor induction: 1x10^4 cervical carcinoma cells injected SC in the left flank on Day 0

Treatment: SC injections (nape of neck) of PBS or 150µg ODN 7909, 1826 or 2137 daily from Day 10 to Day 18.

comparison of murine B16 melanoma cell lines



CpG ODN1585
control ODN 2118
phosphate buffered saline (PBS)

} injected at day 0, 3, 7 and then weekly

CpG ODN1585 (injected at day 0 only)

*** PBS was used only with 0.1 x 10^5 tumor cells**

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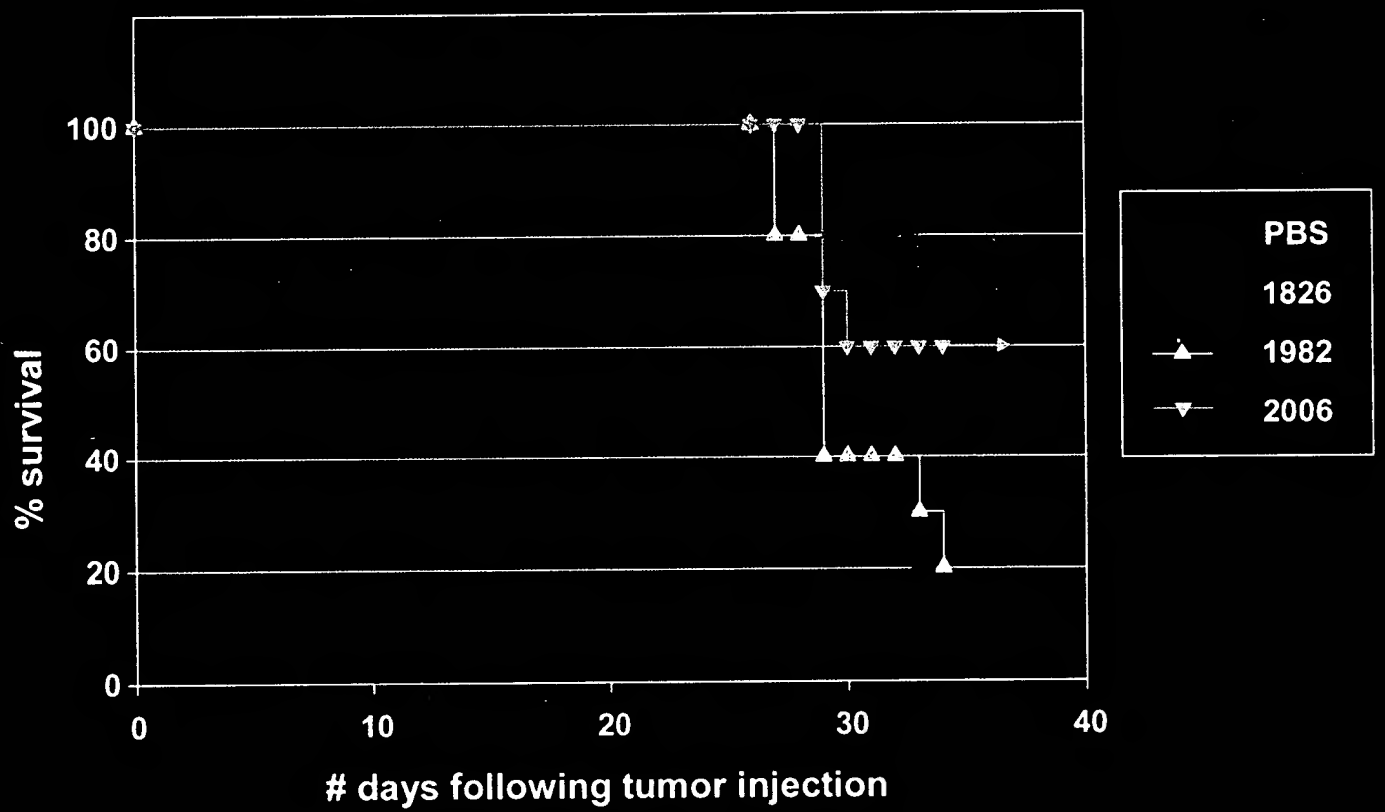
FIGURE 5

MURINE T CELL LYMPHOMA MODEL: CpG MONOTHERAPY

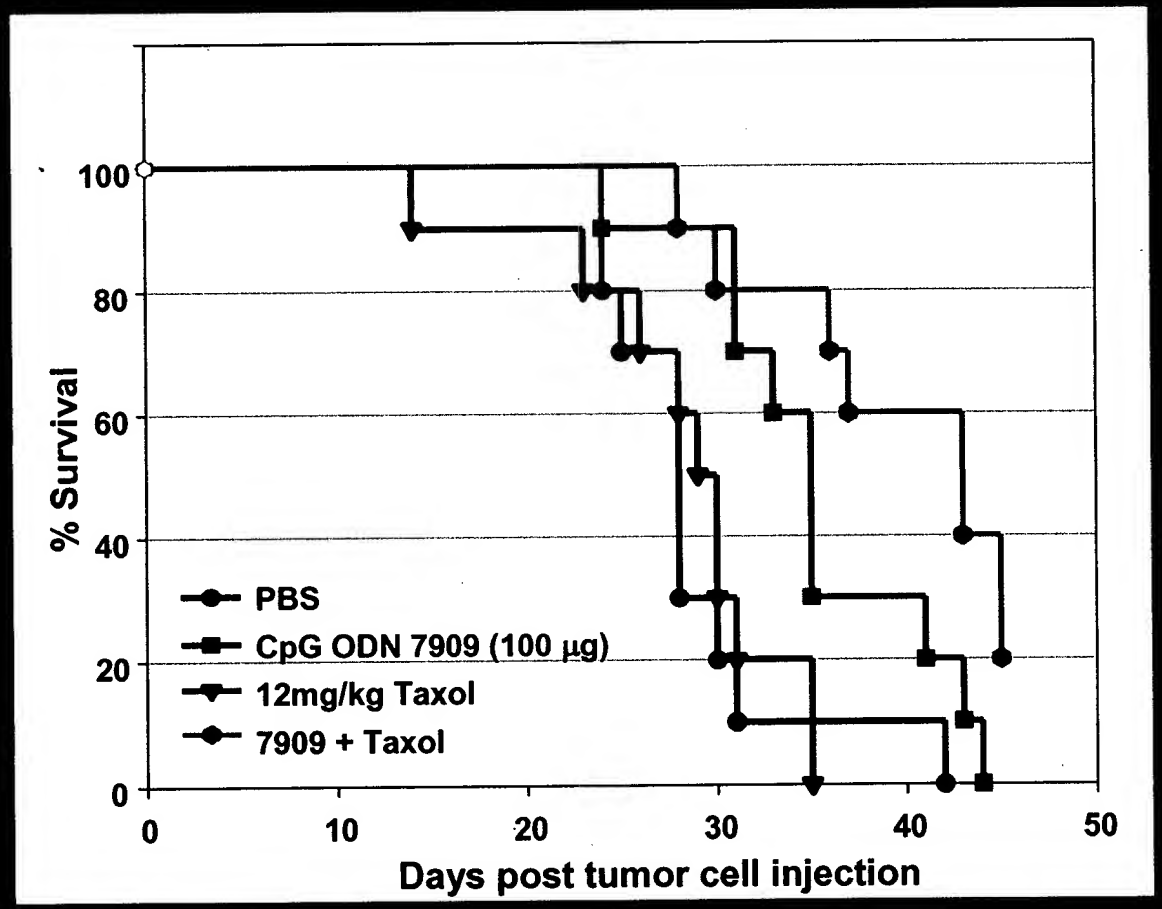


Tumor induction: 1x10^4 EL-4 cells injected SC in the left flank on Day 0

Treatment: SC injections of PBS or 100μg ODN 7909, 1826 or 1982 0n Day 2, 9 and weekly for 2 months.

FIGURE 6

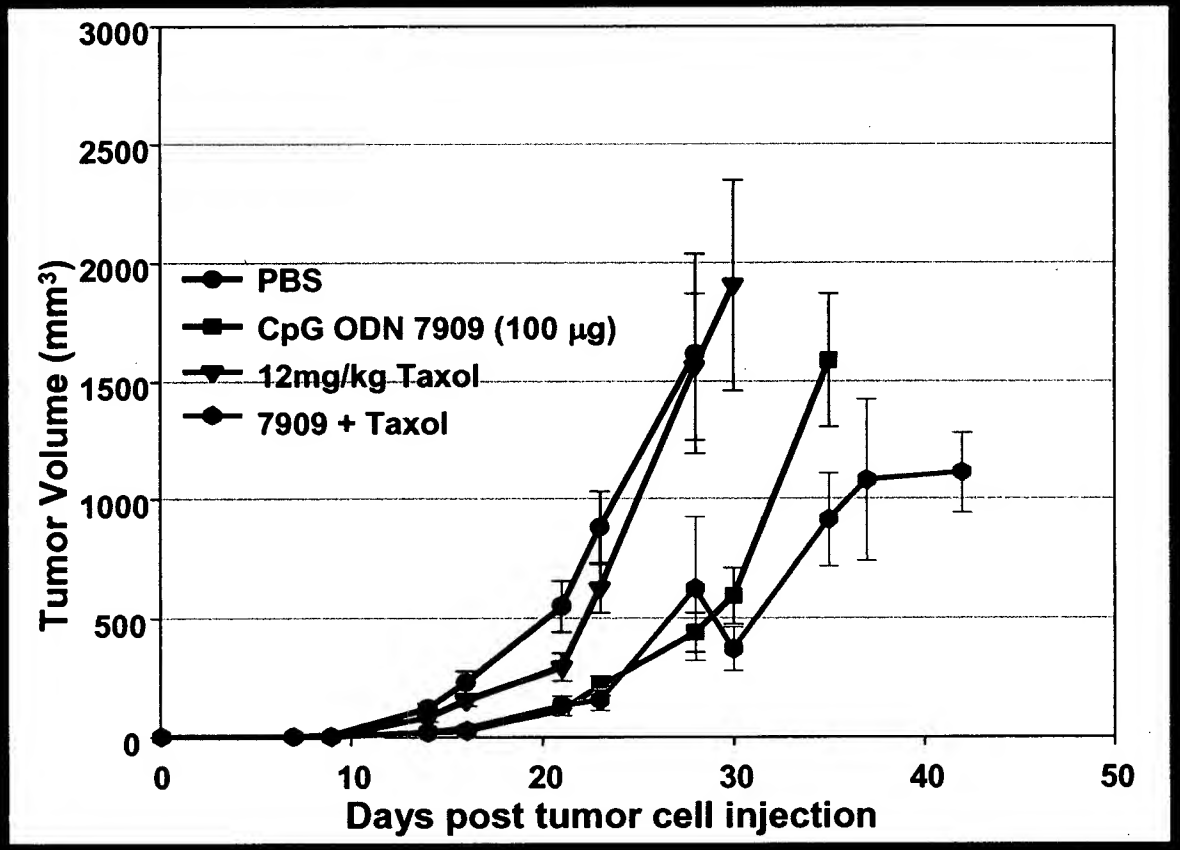
LLC: COMBINATION THERAPY WITH TAXOL + CpG; SURVIVAL OF ANIMALS



Tumor Induction: C57Bl/6 mice, 2 x 10^6 LLC cells SC in left flank on Day 0

Treatment: Taxol (12 mg/kg) IP daily from Day 7 to 11, ODN (100 µg) SC on Days 1, 3, 7 & weekly for 2 months.

LLC: COMBINATION THERAPY WITH TAXOL + CpG; RATE OF TUMOR GROWTH

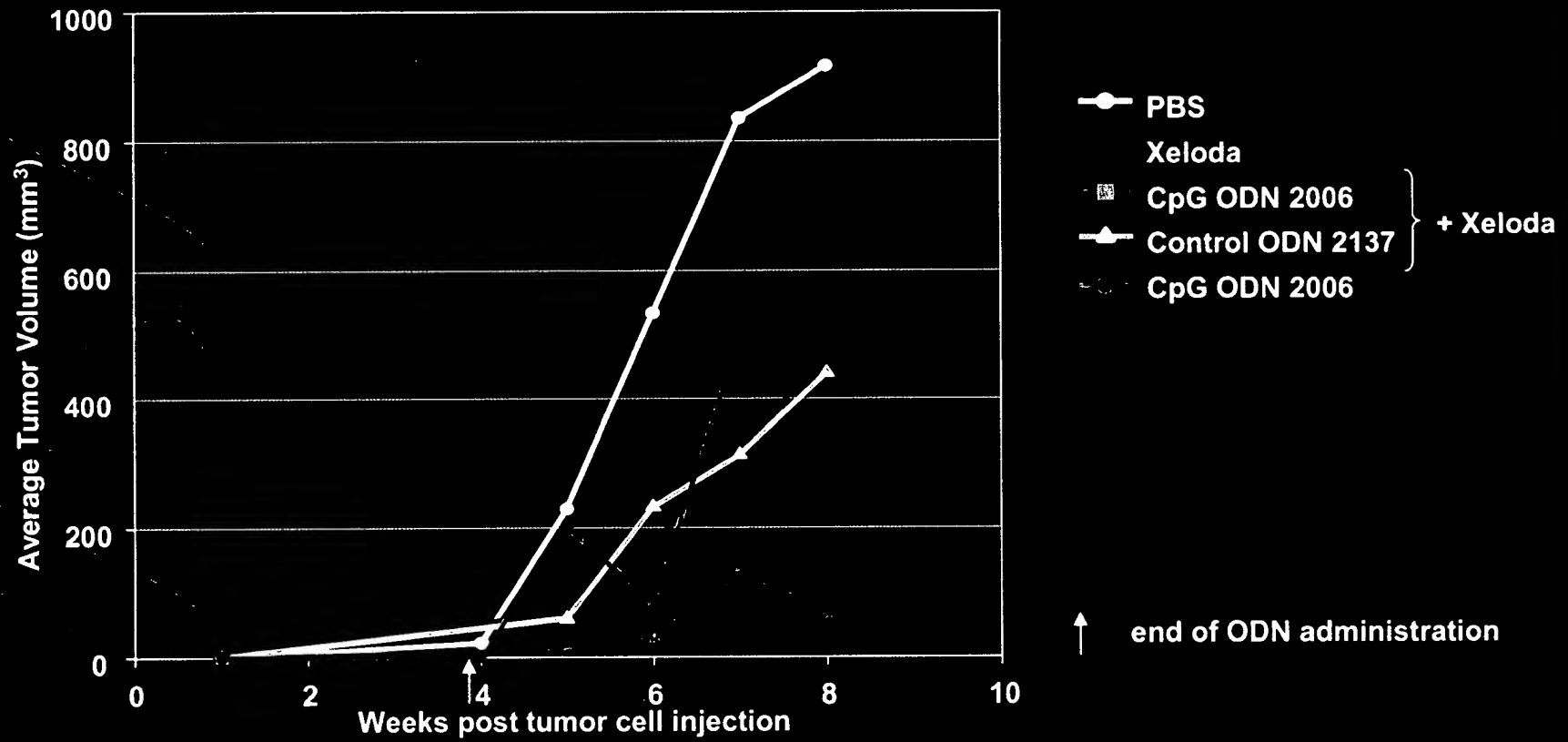


Tumor Induction: C57Bl/6 mice, 2 x 10^6 LLC cells SC in left flank on Day 0

Treatment: Taxol (12 mg/kg) IP daily from Day 7 to 11, ODN (100 µg) SC on Days 1, 3, 7 & weekly for 2 months.

FIGURE 8

CpG + XELODA IN MURINE RENAL CELL CARCINOMA



BALB/C mice injected with $2x10^5$ renca cells SC in the left flank.
Xeloda (359 mg/kg) orally (Day 11-24) and ODN SC into tumor margin weekly from day 10 to 38.

Effects of Intra-muscular CpG Administered With Cyclophosphamide in Mice with Sub-clinical Tumor

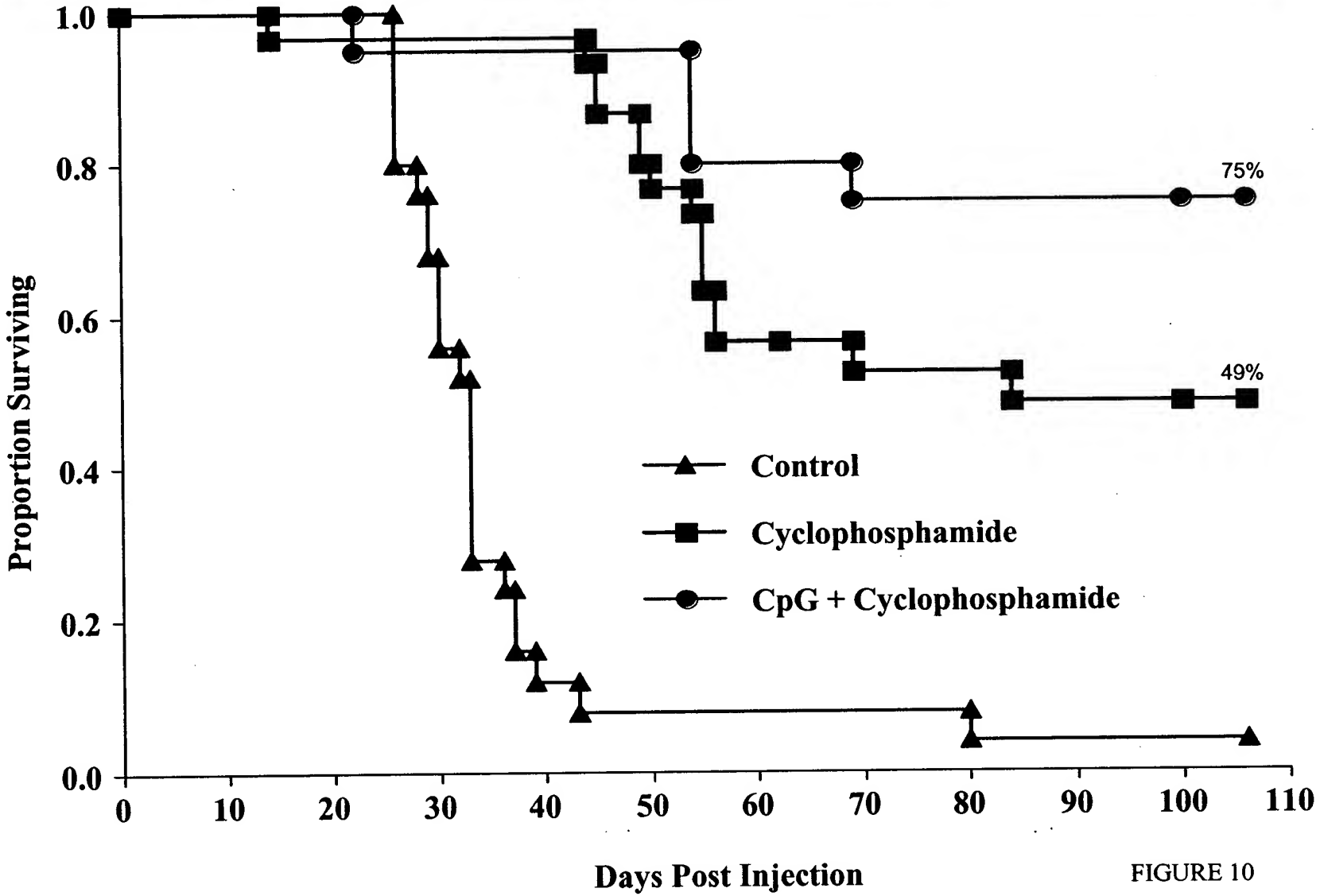


FIGURE 10

Effects of Systemic CpG Administered With Cyclophosphamide in Mice with Sub-clinical Tumors

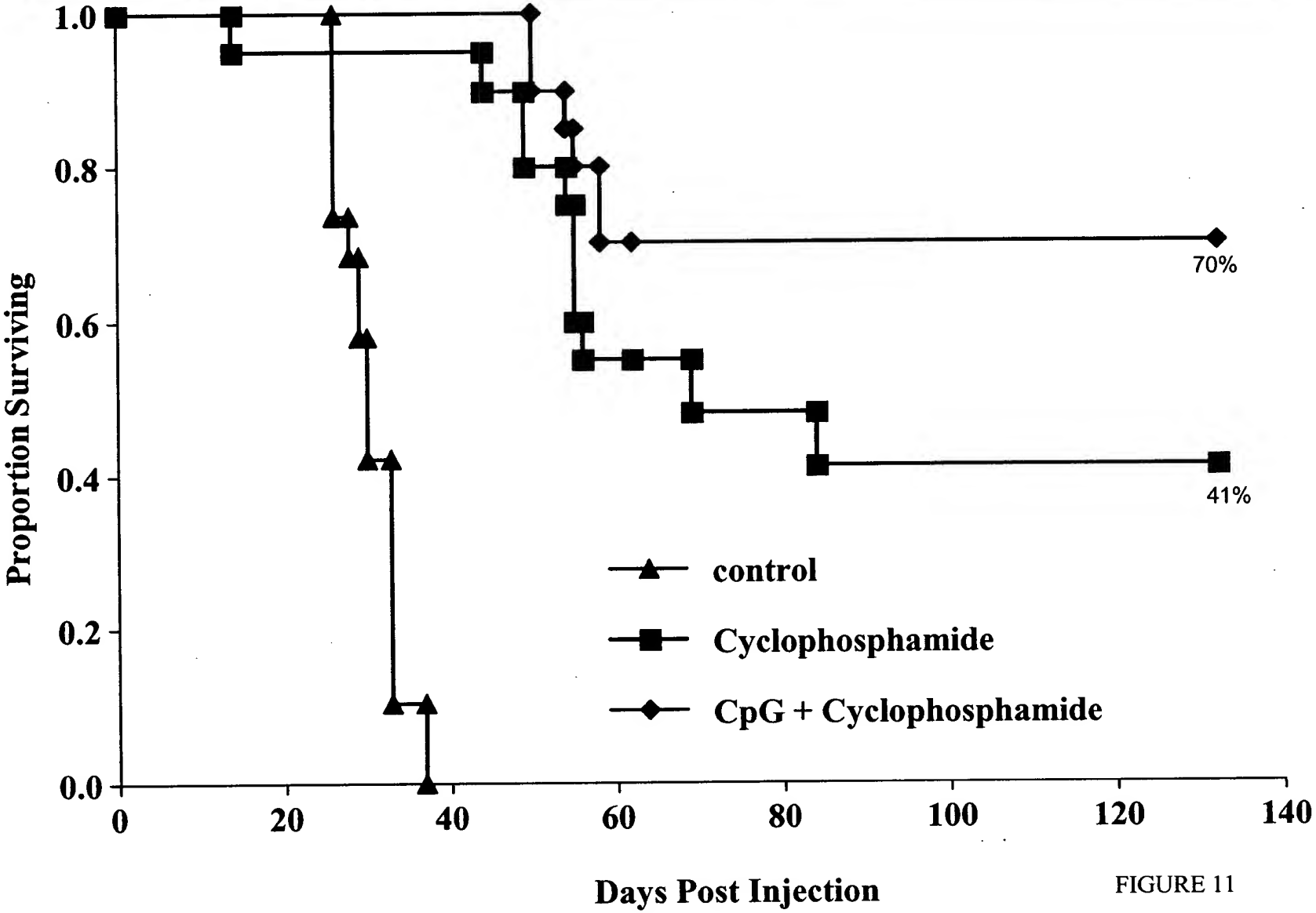


FIGURE 11

Anti-tumor Effect of Systemic CpG Administration With Cyclophosphamide in Mice with Large Tumors

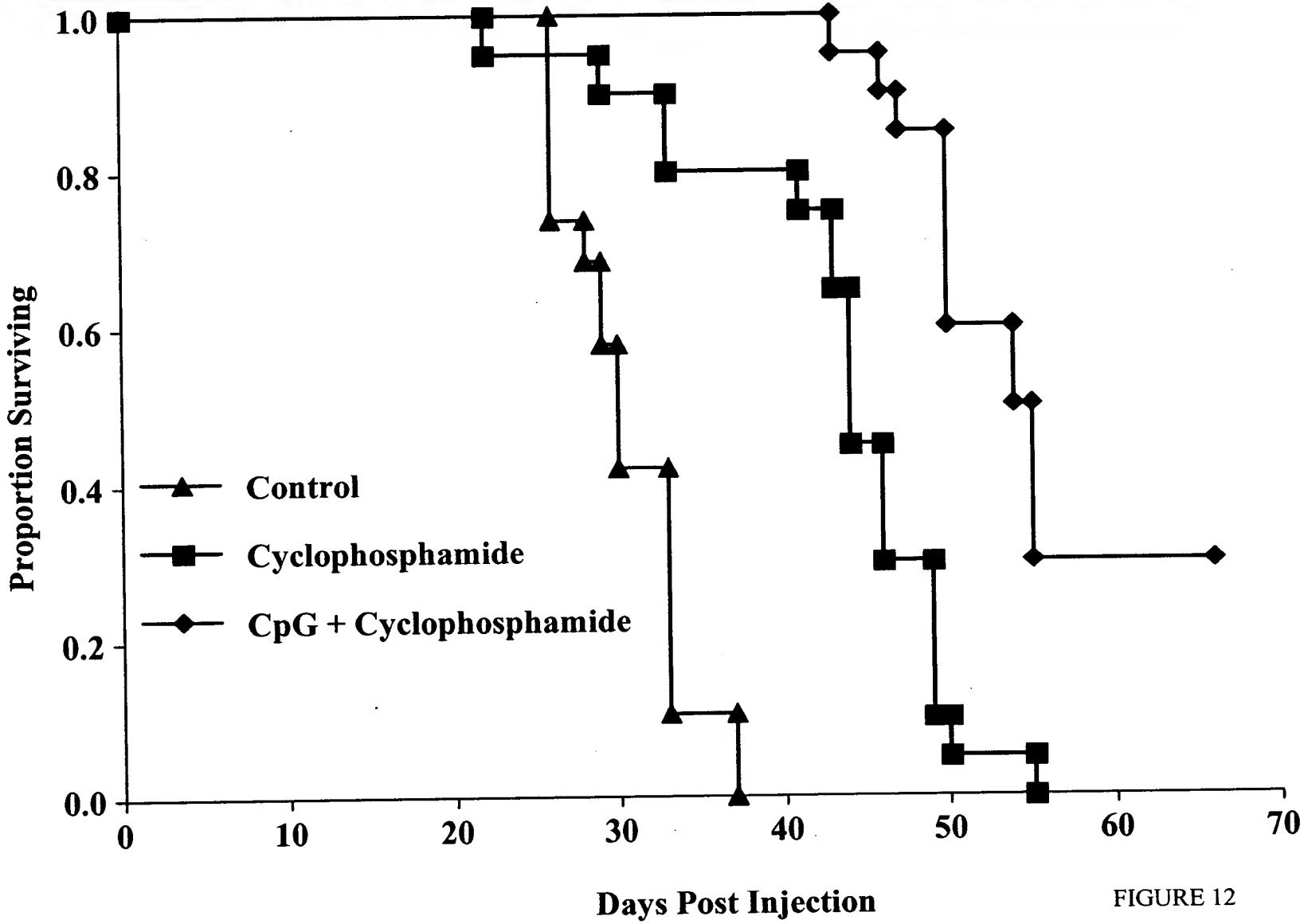


FIGURE 12

Reduced Tumor Size in Mice with Large Tumors Administered Systemic CpG with Cyclophosphamide

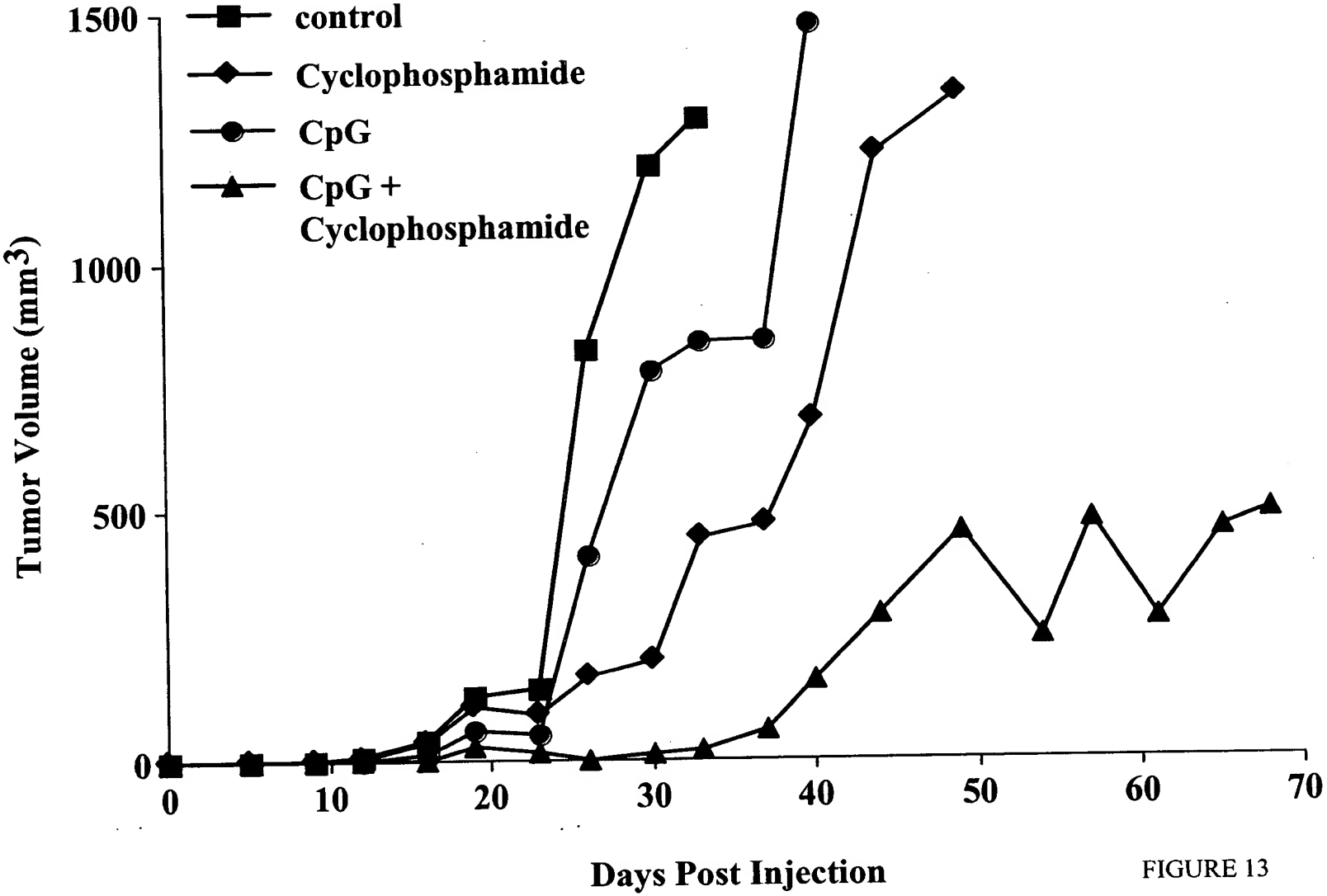


FIGURE 13

Appendix C

Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naive and bone marrow transplant recipients

Bruce R. Blazar, Arthur M. Krieg, and Patricia A. Taylor

Immunostimulatory cytosine-phophate-guanosine (CpG)–containing motifs in bacterial DNA are potent immune system activators. Depending on the bases flanking the CpG motif and on the DNA backbone, CpG oligodeoxynucleotides (ODNs) can induce relatively more B-cell activation or relatively more natural killer (NK)–cell activation. To evaluate their antitumor activities, an NK-optimized ODN (1585) and 2 B-cell–optimized ODNs (1826 and 2006) were compared for their ability to protect naive mice against a lethal acute myelogenous leukemia (AML) challenge. CpG 2006, but not CpG 1585, administered 2 days before the AML challenge, allowed mice to survive more than 100 times a lethal tumor dose. Cell depletion studies showed that protection did not require T or B cells but depended on NK cells and also on an NK-independent mechanism. CpG 2006 protected against AML challenge in both syngeneic and allogeneic bone marrow transplant (BMT) recipients at both early and late time points after transplantation. Although CpG 1585 had no protective effect on its own, it showed a striking synergy with CpG 2006 to induce prolonged survival to AML challenge in allogeneic recipients of T-cell–depleted marrow grafts, exceeding the survival benefit of donor lymphocyte infusion (DLI). When combined with DLI, a synergistic effect was observed in recipients of CpG2006 or 2006 + 1585 with 88% of mice surviving long-term. These data are the first to indicate that the systemic administration of CpG ODNs is a potent means of inducing therapeutic anti-AML innate immune responses in naive and BMT recipients. (Blood. 2001;98:1217-1225)

Introduction

The innate immune system is geared toward providing a rapid response to foreign pathogens by pattern recognition receptors that distinguish prokaryotic from eukaryotic DNA.[1] These receptors specifically bind to unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides, enabling bacteria and other pathogens to stimulate the innate immune system.[2-6] Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs mimic bacterial DNA and can activate immune responses.[2-5] CpG administration results in high T helper 1 (Th1) activity and lower toxicity than complete Freund adjuvant.[7-10] CpG ODNs directly stimulate B lymphocytes to produce interleukin-6 (IL-6) and IL-10[3]; natural killer (NK) cells to produce interferon γ (IFNγ); and monocytes and dendritic cells (DCs) to produce IL-6, IL-12, IL-18, tumor necrosis factor α (TNFα), and IFNα.[11] These soluble factors may activate innate immune cells such as NK cells and also may function to stimulate the adaptive immune system. Stimulation of the adaptive immune system can result in B-cell secretion of immunoglobulin, DC expression of major histocompatibility complex (MHC) class II antigens and costimulatory molecules and T-cell activation from either soluble factors or from binding to activated B cells, DCs, or macrophages.[12-14] Thus, CpG motifs not only engage the innate immune system but also are able to recruit effector cells that comprise the adaptive immune system. As such, CpG ODNs may be exploited as a means of stimulating both the innate and the adaptive immune system to recognize and respond to foreign antigens, as would occur with a viral infection or neoantigens expressed on tumor cells.

Acute myelogenous leukemia (AML) is the most common form of acute leukemia in adults. Intensive chemotherapy regimens have led to remission induction in 70% to 85% of patients. Autologous bone marrow transplantation (BMT) can result in long-term, disease-free survival, although such therapy can be associated with morbidity/mortality, and postremission relapses are frequent.[15-17] Although a graft-versus-leukemia (GVL) effect for AML has been observed in allogeneic bone marrow transplant (alloBMT) recipients and in recipients given delayed lymphocyte infusions (DLIs) to treat relapse after alloBMT,[18,19] the graft-versus-host (GVH) effect that often accompanies the GVL effect is a significant cause of morbidity and mortality. For patients not undergoing BMT or for those receiving autologous BMT, alloresponses as a means to achieving remission are not an option. In these instances, the generation of tumor-specific immune responses requires the tumor cell or an antigen-presenting cell (APC) to present tumor antigens to autologous immune-competent T cells. Tumors usually are poor

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A.M.K. has declared a financial interest in the Coley Pharmaceutical Group, whose product was studied in the present work.

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stimulators of immune responses. Because endogenous T-cell responses are not sufficiently potent as to protect against AML recurrence and alloresponses are associated with graft-versus-host disease (GVHD), alternative approaches to preventing AML recurrence are needed. CpG ODNs are attractive candidates for such purposes.

In autologous and especially alloBMT recipients, T cells generated after BMT may not be fully immunocompetent because of defects in the thymic microenvironment and other types of injury.[20] In contrast to the prolonged period of time that can be required for T-cell immune reconstitution, NK-cell recovery after BMT is rapid.[21-23] NK cells present early after BMT have been shown to be potent effectors against myeloid leukemias.[23] Because CpG ODNs can be potent stimulators of innate immune system effector cells, we reasoned that CpG ODNs might provide a means of achieving an anti-AML response in mice early after BMT prior to reconstitution of a fully functional adaptive immune system. Our data demonstrate that CpG ODNs are highly effective in inducing an anti-AML immune response in naive mice as well as in BMT recipients of either autologous or T-cell–depleted (TCD) allogeneic bone marrow when CpG ODNs are given as early as 2 weeks after BMT, a time period when T-cell reconstitution is poor. The combined administration of CpG ODNs along with mature donor lymphocytes (DLI) completely prevented AML-induced lethality. These data have important clinical ramifications.

Materials and methods

Mice

C57BL/6 (termed B6:H2^b), B10.BR/SgSnJ (H2^k), and B6-severe combined immune deficient (B6-SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were bred and housed in a specific pathogen-free facility in microisolator cages. Mice were used at 8 to 10 weeks of age.

CpG ODNs

Phosphorothioate-modified ODNs and chimeric ODNs were provided by the Coley Pharmaceutical Group (Wellesley, MA). Chimeric ODNs have phosphorothioate linkages between the first 2 bases on the 5′ end and the last 5 bases on the 3′ end with central bases connected by phosphodiester linkages. This arrangement of 5′ and 3′ terminal base modifications previously has been shown to be optimal for in vitro immune stimulation.[24] Pharmacokinetic studies have demonstrated that in vivo such chimeric ODNs containing internal phosphodiester bonds undergo degradation and are cleared from the plasma approximately 10 times faster than corresponding phosphorothioate ODNs.[25] ODN had undetectable endotoxin levels using the Limulus amoebocyte lysate (LAL) assay. ODNs were diluted in Tris-EDTA buffer and further diluted in phosphate-buffered saline for in vivo use. The ODN sequences are CpG 1585, GGGGTCAACGTTGAGGGGGG; CpG 1826, TCCATGACGTTCCTGACGTT; control 1982, TCCAGGACTTCTCTCAGGTT; CpG 2006, TCGTCGTTTTGTCGTTTTGTCGTT; and control 2118, GGGGTCAAGCTTGAGGGGGG. ODNs were injected at 100 μg/dose intraperitoneally once (CpG 1826 or CpG 2006) or twice weekly (CpG 1585). This dose was determined to be optimal for the induction of antitumor responses in several systems involving comparisons of 30, 100, and 300 μg/injection. CpG ODN 1585 was given twice weekly because this ODN has a shorter half-life than the others.[24,25] Approximately 4% of the fully phosphorothioate-modified ODN dose is present in the spleen during the first 24 hours after injection,[26] resulting in a splenic concentration of 4 μg/mL. Because chimeric ODNs such as 1585 are eliminated from the plasma approximately 10 times as fast as fully phosphorothioate-modified ODNs, the spleen concentration is unlikely to exceed 0.4 μg/mL. In one experiment, complete Freund adjuvant was homogenized and given at a dose of 0.2 mL subcutaneously on day −2.

AML challenge

C1498, derived as a spontaneous tumor line from a B6 mouse, was grown in RPMI 1640 (Gibco, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Hyclone, Ogden, UT) as described.[24] C1498 is an MHC class I$^+$II$^-$ AML line.[27] C1498 cells were given intravenously at doses ranging from 2×10^3 to 2×10^6/mouse. Necropsies were performed at the time of death. AML tumor involvement was found in all mice, unless otherwise noted.

Anti-AML responses in naive mice

CpG ODNs were given beginning on day −2 or as late as day 6 until day 26 relative to the injection of C1498 cells (range, 2×10^5 to 2×10^6). In some model systems, administration of CpG ODN must precede challenge with infectious pathogens by 2 days for optimal effect,[28] but, in other model systems, treatment can be delayed for a week or longer.[29] The duration of the CpG ODN immune effects is 2 weeks or more after the last injection.[28] To determine the effectors responsible for the CpG-induced immune response, mice were treated with irrelevant rat immunoglobulin G (IgG) or anti–NK 1.1 (clone PK136)– or anti-CD4 (clone GK1.5)– and/or anti-CD8 (clone 2.43)–depleting monoclonal antibodies (mAbs) given intraperitoneally at a dose of 400 μg each from days −1 and weekly for 4 weeks that routinely results in more than 95% depletion of the targeted cell population for more than 1 week beyond discontinuing of mAb administration.

Anti-AML responses in BMT recipients

To generate syngeneic BMT recipients, B6 mice were irradiated with 8.0 Gy total body irradiation (TBI) on day −1 and reconstituted with 5×10^6 B6 bone marrow cells on day 0. CpG ODNs were administered beginning on day 63 and ending on day 90 (CpG 1585) or day 92 (CpG 2006) after BMT. C1498 was given intravenously on day 80 after BMT. Cohorts received 2 subcutaneous vaccines of irradiated C1498 cells (10^7) given on days 66 and 73 or no immunizations.

To determine the effects of CpG ODNs given later after alloBMT, B6 recipients were irradiated with 8.0 Gy TBI on day −1, reconstituted with B10.BR TCD bone marrow (2×10^7). CpG ODNs were administered during the time period of days 72 to 112, and C1498 cells (2×10^5) were given on day 86 after BMT. To assess the effects of CpG ODNs early after BMT, CpG ODNs were injected from days 20 to 62 after BMT, and C1498 cells (2×10^5) were given to recipients on day 36 after BMT. As a more rigorous test, CpG ODNs were injected from days 15 to 42 after BMT, and C1498 cells (2×10^5) were given on day 17 after BMT. AlloBMT recipients were monitored daily for survival and clinical appearance and weighed twice weekly. All available animals were examined by necropsy for evidence of GVHD and tumor.[30]

To assess the effects of CpG ODNs on DLI-mediated GVHD and GVL, B6 recipients were given B10.BR TCD bone marrow as described above. On day 17 after BMT, C1498 cells (2×10^5) were given under the cover of CpG ODNs that were administered from days 15 to 42 after BMT. On day 21 after BMT, splenocytes (25×10^6) from B10.BR donors[30] were infused.

Flow cytometry

Cells were incubated with anti-FcR mAb and then mAb directed toward CD4 or CD8, CD19, and Mac1. Cells were analyzed by 2- or 3-color flow cytometry by using fluorescein isothiocyanate-, phycoerythrin-, or biotin-conjugated (along with SA-peridinin chlorophyll protein) mAb (Pharmingen). Irrelevant mAb control values were subtracted from values obtained with relevant values, using a FACScalibur (Becton Dickinson, Mountain View, CA). Forward and side scatter settings were gated to exclude red cells and debris, and 10^4 cells were analyzed for each determination.

Cytokine analysis

B6 splenocytes (10^6/mL) were plated in RPMI 1640 with 10% fetal bovine sera. ODNs were added at 0.3 to 3.0 μg/mL. Supernatants were collected for enzyme-linked immunosorbent assay analysis (IL-6, IL-12, IFNγ, TNFα: all sensitivities < 50 pg/mL).

Table 1. Effect of cytosine-phosphate-guanosine oligodeoxynucleotides on proinflammatory cytokine production

CpG ODN	Concentration (μg/mL)	IFNγ	IL-6	IL-12	TNFα
1585	0.3	281 ± 66	205 ± 68	878 ± 64	< 50
1585	1.0	627 ± 106	2 580 ± 136	6 531 ± 123	767 ± 218
1585	3.0	1 674 ± 368	5 501 ± 592	11 487 ± 507	3 486 ± 837
1826	0.3	323 ± 36	6 174 ± 1 056	9 811 ± 65	308 ± 237
1826	1.0	423 ± 72	16 105 ± 3 144	10 041 ± 963	868 ± 186
1826	3.0	*884 ± 131*	17 163 ± 1 918	7 476 ± 1 639	1 985 ± 594
2006	0.3	233 ± 108	829 ± 26	3 605 ± 407	252 ± 418
2006	1.0	337 ± 124	5 033 ± 242	7 755 ± 734	*243 ± 113*
2006	3.0	*684 ± 253*	10 846 ± 587	7 895 ± 1 413	*1 404 ± 367*
1982	0.3	475 ± 175	*423 ± 620*	*395 ± 33*	< 50
1982	1.0	*191 ± 7*	*316 ± 116*	*130 ± 152*	*51 ± 258*
1982	3.0	*381 ± 44*	*554 ± 179*	*3 082 ± 297*	1 468 ± 630
2118	0.3	*500 ± 9*	*283 ± 77*	*546 ± 37*	< 50
2118	1.0	*243 ± 46*	*324 ± 61*	*454 ± 14*	*< 50*
2118	3.0	*434 ± 36*	*731 ± 116*	*386 ± 155*	*897 ± 152*

B6 splenocytes were incubated for 24 hours with the indicated ODNs. After 24 hours, supernatant was collected for cytokine analysis by enzyme-linked immunosorbent assay as described in "Materials and methods." Triplicate samples were analyzed. Cytokine values are in pg/mL. Data are expressed as mean ± 1 SD. A value of < 50 pg/mL indicates that the mean value was at the limits of detection of the assay. These data were reproduced in a replicate experiment.

Underlined values indicate cytokine values that are significantly ($P < .05$) higher than those observed with CpG 1585 at the corresponding ODN concentration. Values in italics indicate cytokine values that are significantly ($P < .05$) lower than those observed with CpG 1585 at the corresponding ODN concentration.

CpG indicates cytosine-phosphate-guanosine; ODN, oligodeoxynucleotide; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Statistical analyses

Group comparisons of continuous data were made by Student *t* test. Survival data were analyzed by life table methods, using the Mantel-Peto-Cox summary of chi-square. Actuarial survival and relapse rates are shown. *P* values ≤ .05 were considered significant.

Results

Delineation of ODN with distinct profiles of immune activation

In previous studies we reported that ODNs containing particular arrangements of CpG motifs with a phosphodiester backbone flanked by polyG motifs with a nuclease-resistant phosphorothioate backbone had dramatically enhanced NK-stimulating effects compared with ODNs with all-thioate backbones.[31] We have recently reported that the optimal CpG motif for activating murine B cells is GACGTT, whereas that for activating human B cells is GTCGTT.[32,33] To investigate the immune-stimulatory effects, in vitro assays were performed using representative ODNs from these 3 classes: NK-optimized ODN 1585, mouse B-cell stimulating-optimized ODN 1826, and human-stimulating ODN 2006. Each ODN stimulated splenic production of proinflammatory cytokines (TNFα, IFNγ, IL-6, and IL-12) (Table 1). Although IL-12 was induced to a similar degree by all 3 ODNs at high ODN concentrations, IL-12 production at lower ODN concentrations showed the following hierarchy: CpG 1826 > 2006 > 1585. On the basis of achievable in vivo concentrations, relevant comparisons would be CpG 1585 at 0.3 μg/mL versus CpG 2006 at 3.0 μg/mL. CpG 1585 was a more potent inducer of IFNγ and TNFα production than CpG 1826 or 2006. Consistent with IFNγ and TNFα expression, CpG 1585 is a more potent inducer of NK-cell function than the other ODNs (Z. Ballas, personal communication, 2001). In contrast, CpG 1826 and 2006 were more potent inducers of IL-6 production as compared with CpG 1585. B-cell proliferation roughly correlated with induction of IL-6 expression (data not shown). These data all were reproduced fully in a second experiment. We conclude that CpG 2006 is a more potent inducer of the proinflammatory cytokines IL-12, IL-6, and IFNγ at biologically achievable doses as compared with CpG 1585.

Administration of CpG ODNs in vivo is highly effective in stimulating immune responses to AML cells

The immune response to a low burden of C1498 cells (≤ 3×10^4) is dependent on NK cells, whereas resistance to a higher tumor burden (≥ 10^5) is T-cell dependent.[27] To determine whether CpG ODNs might stimulate an immune response to a higher tumor burden, naive B6 mice were given CpG ODNs selected as potent stimulators of NK-cell function (CpG 1585) or as potent stimulators of B-cell and DC function (CpG 1826 or CpG 2006). In vitro, C1498 proliferation was not impaired by exposure to high concentrations (100 μg/mL) of CpG 1585 or CpG 2006 ODNs (CpG 1826 was not tested) (not shown). B6 recipients challenged with C1498 cells (2×10^5/mouse) succumbed within 5.5 weeks to tumor (Figure 1). Complete Freund adjuvant did not significantly affect survival. Recipients given CpG 1585 had a significant ($P < .008$) extension in survival, although all succumbed to tumor by 7.5

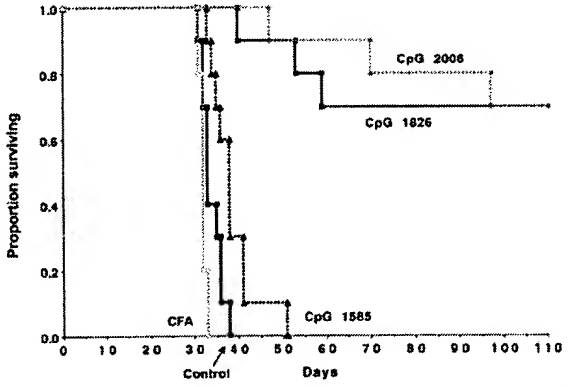

Figure 1. CpG ODN administration can protect naive mice against AML-induced lethality. B6 mice (n = 10 per group) were treated with the indicated CpG ODN (100 μg/dose) intraperitoneally beginning on day −2 prior to intravenous C1498 (2×10^5 cells/mouse) infusion according to the schedule described in "Materials and methods." CpG ODN-treated mice had a significant ($P < .008$) increase in survival rates as compared with nontreated or complete Freund adjuvant-treated controls. Recipients of CpG 1826 or 2006 ODNs had comparable survival that was superior ($P \leq .001$) to CpG 1585-treated recipients.

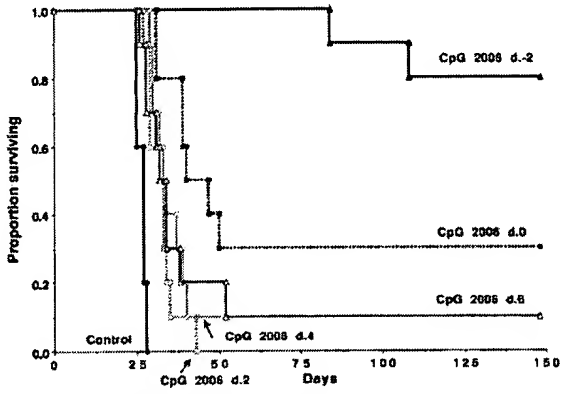

Figure 2. CpG 2006 ODN is more effective when administration is initiated before AML challenge. B6 mice (n = 10 per group) were treated with weekly CpG 2006 ODN initiated as indicated, relative to C1498 (2×10^5 cells/mouse) infusion. CpG ODN-treated recipients survived significantly ($P < .003$) longer than nontreated controls, regardless of the schedule used. Mice receiving CpG 2006 beginning on day −2 survived significantly ($P < .001$) longer than all other groups; those that received CpG 2006 beginning on day 0 survived significantly ($P < .05$) longer than all groups except the day −2 group.

weeks after the challenge. In contrast, CpG 1826 or CpG 2006 given beginning on day −2 before C1498 challenge protected 70% of mice from a lethal AML dose (Figure 1).

CpG 2006 rather than CpG 1826 was selected for further study because a similar effect was observed with these 2 ODNs, and CpG 2006 is immune stimulatory in humans and mice.[32] Although all controls died within 4 weeks of C1498 challenge (2×10^5 cells), 80% of the recipients treated with CpG 2006 beginning on day −2 survived the 5-month period (Figure 2). CpG 2006 given on day 0 of the C1498 challenge rescued 30% of mice. A delay in CpG administration of 2, 4, or 6 days after the C1498 challenge significantly ($P \leq .001$) prolonged survival as compared with controls, although 10% or less survived long-term. A greater degree of protection required that CpG ODNs were administered before or with the C1498 challenge.

To quantify the magnitude of the CpG 2006 effect, recipients were challenged with various C1498 doses, ranging from 2×10^4 to 2×10^6 cells (Figure 3). A cohort was given CpG 2006 and

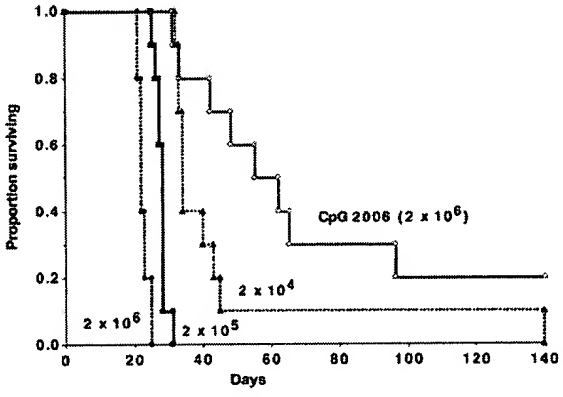

Figure 3. CpG 2006 ODN administration reduces AML tumorigenicity by more than 100-fold. B6 mice (n = 10 per group) were challenged with various C1498 doses, as indicated. A cohort of mice was treated with CpG 2006 ODN, beginning on day −2. Mice receiving CpG 2006 survived significantly ($P \leq .03$) longer than all groups, including those that received 100-fold fewer C1498 cells (2×10^4 cells/mouse).

2×10^6 cells. In controls, a dose-response effect of C1498 cells was evident, as measured by time to mortality. None of the mice survived a challenge with 2×10^4 cells or 2×10^5 cells. In contrast, 20% of CpG 2006-treated recipients of 2×10^6 C1498 cells survived long-term, significantly ($P = .002$) higher than recipients of 100-fold fewer cells.

To determine which effector cells were required for the antitumor effects mediated by CpG administration, CpG 2006-treated B6 recipients of C1498 cells (6×10^5) were given irrelevant mAb or mAbs to deplete T cells or NK cells (Figure 4A). In contrast to irrelevant mAb-treated controls that died of tumor by 25 days after infusion, CpG treatment significantly delayed tumor mortality by 50 days with all succumbing to tumor by 75 days after infusion ($P = .008$). CpG-treated mice receiving 6×10^5 C1498 cells had a modest but not statistically significant longer mean survival time ($P = .10$) as compared with controls receiving 30-fold fewer C1498 cells, indicating a high level of antitumor activity conferred by CpG administration. Depletion of NK cells significantly impaired survival in CpG-treated recipients

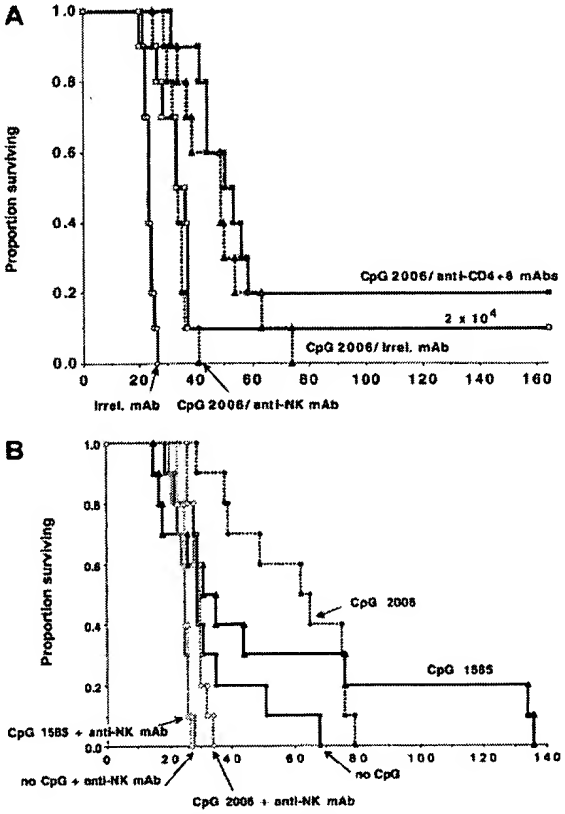

Figure 4. NK-cells but neither T nor B cells are obligatory for optimal CpG ODN-induced anti-AML responses. B6 (A) or B6-SCID (B) mice (n = 10 per group) were treated with CpG 1585 or 2006, beginning on day −2 prior to the infusion of C1498 cell (A: 6×10^5 cells/mouse unless otherwise indicated; B: 2×10^5 cells/mouse). Cohorts of mice were given irrelevant, anti-NK or anti-CD4+CD8 mAbs, as indicated, according to "Materials and methods." (A) CpG 2006 administration reduced tumorigenicity as compared with controls ($P = .0008$). Depletion of NK cells ($P = .0054$) but not T cells ($P = .14$) significantly reduced the anti-AML efficacy of CpG 2006, although CpG-treated NK-depleted recipients survived significantly ($P = .0006$) longer than non–CpG-treated controls. (B) B6-SCID mice treated with CpG 2006 but not CpG 1585 survived significantly longer after C1498 challenge than controls ($P = .00$ and .16, respectively). NK-depleted B6-SCID mice treated with CpG 2006 but not CpG 1585 survived significantly longer after C1498 challenge than controls ($P = .0008$ and .36, respectively).

($P = .0054$), although CpG-treated, NK-depleted mice survived longer than controls ($P = .0006$). In contrast, pan-TCD did not adversely affect survival ($P = .14$). Additionally, CpG-treated recipients depleted of both NK and T cells had similar survival to CpG-treated recipients depleted of NK cells alone (not shown). These data were reproduced in other experiments using a lower tumor cell dose (2×10^5) (not shown). Together, these studies indicate that the tumor protection conferred by CpG administration is mediated primarily but not exclusively by NK cells.

Long-term CpG 2006-treated B6 survivors of C1498 challenge (110-148 days) were rechallenged with C1498 cells at the same dose as used for the initial challenge (2×10^5 cells). Despite the potent CpG 2006-induced anti-AML effects, pooled data in 2 experiments revealed that all 12 CpG 2006-treated recipients succumbed to AML lethality within 32 days after rechallenge, a time course similar to that observed with the initial challenge and with concurrent controls (not shown). Subsequent experiments will be designed to determine whether CpG given at AML rechallenge will be as effective as treatment at the time of initial AML challenge. Together, these data indicate a lack of memory-cell responses in CpG-treated recipients and are consistent with NK cells as the major effector cells in CpG 2006-mediated anti-AML responses.

Studies were performed in CpG-treated B6-SCID mice to determine whether CpG ODNs could provide an anti-AML effect in T- and B-cell–deficient mice. B6-SCID mice were treated with CpG 2006 or CpG 1585 and challenged with C1498 cells (2×10^5). In B6-SCID mice, known to have heightened NK activity, CpG 2006 but not CpG 1585 significantly delayed the time to AML-induced lethality (Figure 4B). NK-cell depletion of B6-SCID mice reduced the time to AML-induced lethality as compared with non–NK-depleted controls ($P = .006$), indicating that endogenous NK function in B6-SCID mice provided some anti-AML immune responses (Figure 4B). NK-cell depletion completely abrogated the effect of CpG 1585 in B6-SCID mice. In contrast, although NK-cell depletion largely eliminated the anti-AML effect of CpG 2006, NK-cell depleted, CpG 2006-treated B6-SCID mice survived significantly longer than NK-cell–depleted controls ($P = .0008$), suggesting that cell types other than T, B, or NK cells stimulated by CpG 2006 contributed to the anti-AML response. The most likely candidate populations are DCs and monocytes, known to be activated by CpG 2006.

CpG 2006 but not CpG 1585 induces anti-AML responses in syngeneic BMT recipients

Immunotherapy may be most useful in the setting of minimal residual disease as might occur after BMT. However, after BMT, the adaptive immune system is slow to be restored. Initial studies were performed in syngeneic BMT recipients that were challenged with C1498 (10^5) cells on day 80 after BMT (Figure 5A). Cohorts of mice were treated with CpG 2006 or CpG 1585, beginning on day 64 after BMT or were immunized with irradiated C1498 cells (10^7 on days 66 and 73 after BMT) prior to AML challenge. Another control group received a lower C1498 dose (10^4), which is nonlethal to non-BMT recipients. Consistent with post-BMT immune dysfunction, recipients challenged with a typically nonlethal dose of 10^4 C1498 cells succumbed to leukemia within 2 months after the challenge. BMT recipients challenged with 10^5 cells died faster, succumbing by 1.5 months after challenge. In contrast, 50% of CpG 2006-treated recipients survived the C1498 cell (10^5) challenge long-term (> 4.5 months after challenge), indicating a more than 10-fold reduction in AML-induced lethality.

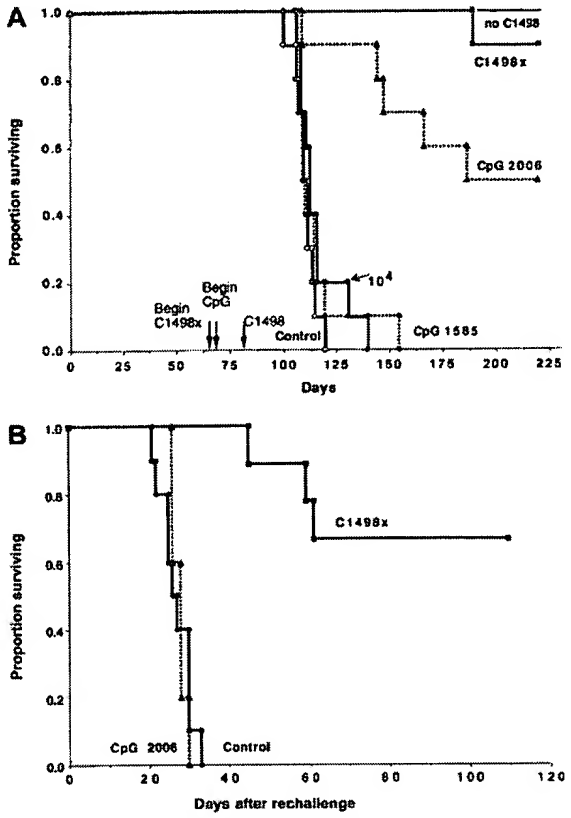

Figure 5. CpG 2006 treatment prolongs the survival of syngenic BMT recipients challenged with AML cells 2.5 months after BMT but does not induce a memory cell response. Lethally irradiated B6 recipients were reconstituted with B6 bone marrow cells (n = 10 per group). Cohorts of mice, as indicated, received CpG 1585 or 2006 ODNs beginning on day 64 and continuing through day 90 (CpG 1585) or day 92 (CpG 2006) after BMT. A separate cohort received irradiated tumor vaccines (termed C1498x) given subcutaneously on days 66 and 73 after BMT. Mice were challenged with C1498 (10^5/mouse) or, as indicated, no C1498 cells or C1498 (10^4/mouse). All surviving mice in the indicated groups were challenged with C1498 cells (10^5/mouse) on day 219 after BMT to assess memory cell response. (A) Although CpG 1585-treated recipients did not protect mice against C1498 challenge ($P = .21$ versus control), CpG 2006 was effective ($P = .00053$ versus control), resulting in more than a 10-fold reduction in tumorigenicity when comparing outcome to recipients of C1498 at a dose of 10^4 cells/mouse ($P = .00074$). (B) C1498x but not CpG 2006 induced a memory cell response as compared with control BMT recipients not previously challenged with C1498 cells ($P = .00015$ and .46, respectively).

Irradiated cellular vaccines were highly effective in inducing AML resistance with 90% of BMT recipients surviving long-term ($P = .023$ versus CpG 2006).

Surviving recipients were challenged with C1498 cells (10^5) on day 219 after BMT and were compared with a group of concurrently challenged BMT controls that had not received prior exposure to C1498 cells (Figure 5B). Long-term survivors initially treated with CpG 2006 had no evidence of a memory cell response, as these recipients died at an identical rate as concurrently challenged BMT controls. In contrast, 67% of recipients immunized with irradiated cellular vaccines survived more than 3.5 months after rechallenge. Thus, irradiated cellular vaccines but not CpG 2006 treatment is able to induce a demonstrable anti-AML memory cell response in syngeneic BMT recipients.

To determine whether CpG 2006 could induce anti-AML responses at early times after BMT, syngeneic BMT recipients were challenged on day 14 after BMT with C1498 cells at doses varying from 2×10^3 to 10^5 (Figure 6). Cohorts received CpG

2006 beginning on day 12 after BMT. At that time, splenic analyses were performed on representative mice (n = 5 per group) to determine the status of immune reconstitution and whether CpG 2006 had influenced these parameters by the day of C1498 challenge. Comparable splenic cellularity and very low but similar absolute numbers of T cells (range, 1.0-1.6 × 10^6 in individual mice) were noted in these 2 groups, consistent with peripheral T-cell deficiency. Recipients challenged with 10^5 C1498 cells succumbed to leukemia within 1 month after challenge. Although CpG 2006 delayed the time to mortality in recipients of 10^5 cells as compared with nontreated controls ($P = .002$), all succumbed to leukemia by 8 weeks after challenge. Fifty percent of recipients of 10^4 cells survived long-term and there was no mortality in recipients of 2 × 10^3 cells. Eighty percent of CpG 2006-treated recipients of 10^4 cells survived long-term ($P = .13$ versus controls). Because recipients of 10^4 cells had a superior survival to CpG-treated recipients of 10^5 cells ($P = .046$) and because recipients of 2 × 10^3 cells had a superior survival to CpG-treated recipients of 10^4 cells ($P = .040$), these data are consistent with a less than the 10-fold reduction observed when CpG 2006 was administered later after BMT. Regardless of the degree in reduction in the tumorigenicity of C1498, it is clear that CpG 2006 can augment the anti-AML response of syngeneic BMT recipients before the adaptive immune system can be fully reestablished.

AlloBMT recipients of full MHC-disparate TCD bone marrow require longer periods of time for functional immune reconstitution as compared with syngeneic BMT recipients. To determine whether the beneficial effects of CpG ODN early after syngeneic BMT would translate into an alloBMT setting, irradiated B6 recipients were reconstituted with B10.BR TCD bone marrow and then challenged with C1498 (2 × 10^5 cells) on day 36 after BMT (Figure 7). Cohorts received no CpG ODN, CpG 1585, or CpG 2006 beginning day 20 after BMT. A cohort of nontreated controls received a lower C1498 dose (2 × 10^4 cells). All recipients of

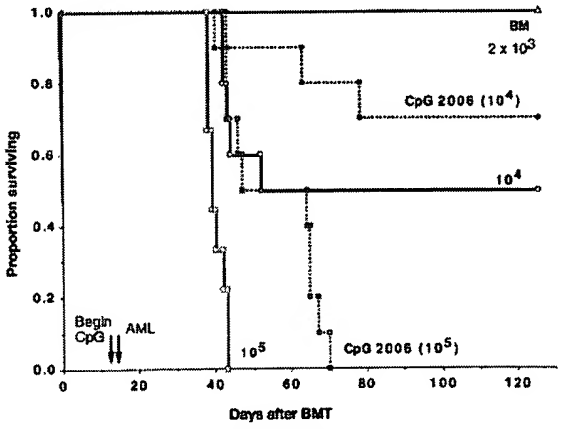

Figure 6. CpG 2006 treatment prolongs the survival of syngenic BMT recipients challenged with AML cells early (day 17) after BMT. Lethally irradiated B6 recipients were reconstituted with B6 bone marrow cells (n = 9-10 per group). Cohorts of mice, as indicated, received CpG 2006 ODNs beginning on day 12 and continuing through day 45 after BMT. C1498 cells were infused at the indicated cell doses on day 14 after BMT. A separate cohort received no C1498 cells (termed BM). CpG 2006-treated recipients of C1498 cells (10^5/mouse) survived longer than controls ($P = .0025$), whereas those that received C1498 cells (10^4/mouse) had a similar survival rate as compared with controls (70% versus 50%; $P = .13$). The decrease in tumorigenicity induced by CpG 2006 was less than 10-fold because CpG 2006-treated recipients of C1498 cells at 10^5 had a poorer survival than control recipients of 10^4 cells ($P = .046$). In addition, CpG 2006-treated recipients of C1498 cells at 10^4 had a poorer survival than control recipients of 2 × 10^3 cells ($P = .04$).

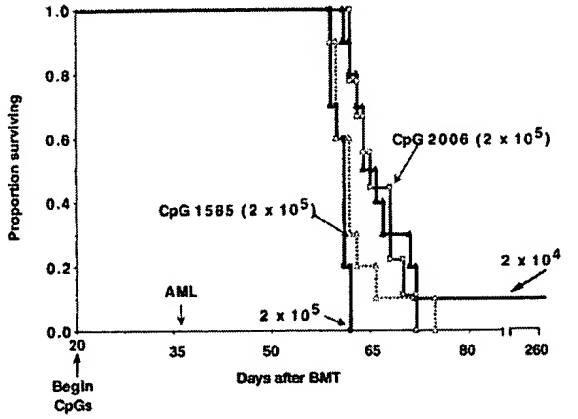

Figure 7. CpG 2006 treatment prolongs the survival of recipients of allogeneic TCD bone marrow challenged with AML cells early (day 36) after BMT. Lethally irradiated B6 recipients were reconstituted with B10.BR TCD bone marrow (n = 9-10 per group). Cohorts of mice received no treatment, CpG 1585, or CpG 2006 ODNs. ODN treatment was initiated on day 20 and continued through day 65. On day 36, recipients were given C1498 cells at the indicated cell dose. Recipients treated with CpG 1585 or CpG 2006 had a prolonged survival compared with controls receiving 2 × 10^5 cells ($P = .045$ and .001, respectively). Recipients given CpG 1585 and C1498 cells at a dose of 2 × 10^5 cells survived for a shorter duration, whereas those receiving CpG 2006 survived for a comparable duration than controls receiving 10-fold fewer C1498 cells (2 × 10^4) ($P = .049$ and .34, respectively). On the basis of these data, CpG 2006 resulted in an approximate 10-fold reduction in tumorigenicity.

2 × 10^5 C1498 cells died within 4 weeks, and 90% of recipients of 10-fold lower cells died within 5.5 weeks after AML infusion. As compared with controls, CpG 1585-treated recipients of 2 × 10^5 C1498 cells had a slight increase in survival ($P = .045$). However, all recipients succumbed within 6 weeks of challenge, and survival was shorter than controls given 2 × 10^4 cells ($P = .049$). As compared with controls, CpG 2006-treated recipients challenged with 2 × 10^5 cells survived longer ($P = .001$) with all mice succumbing approximately 5 weeks after tumor challenge. The degree of tumor reduction was estimated to be about 10-fold because survival of CpG 2006-treated recipients given 2 × 10^5 cells was similar to controls given 2 × 10^4 cells ($P = .34$).

Studies have shown that the innate system returns earlier than the adaptive immune system in recipients of allogeneic TCD bone marrow grafts.[20,23] A significant correlation between return of IL-2–activated NK cells with antimyeloid leukemia cytolytic potential by 3 weeks after BMT and the reduced risk of relapse has been reported.[23] Because CpG ODNs can activate the innate immune system, we modified the model to analyze the effects of CpG ODNs on anti-AML immune responses early after alloBMT prior to reconstitution of the adaptive immune system. Irradiated B6 recipients were reconstituted with B10.BR TCD bone marrow. Recipients were challenged with 2 × 10^5 C1498 cells on day 17 after BMT. Cohorts were given no CpG ODNs, CpG 1585, CpG 2006, or both CpGs (Figure 8A). Additional cohorts received a single DLI dose (25 × 10^6 cells) on day 21, the typical day of DLI administration in this model,[28] alone or in combination with CpG ODNs (Figure 8B). Control and CpG 1585-treated recipients of C1498 died at a similar rate with all succumbing within 31 to 38 days after challenge (Figure 8A). In contrast, CpG 2006-treated recipients survived significantly longer than controls ($P = .015$), requiring 6 months after challenge for uniform lethality. Three (33%) of 9 recipients that were autopsied had no gross evidence of tumor. Combined administration of CpG 1585 + 2006 ODNs resulted in 20% long-term survival (> 8 months after challenge),

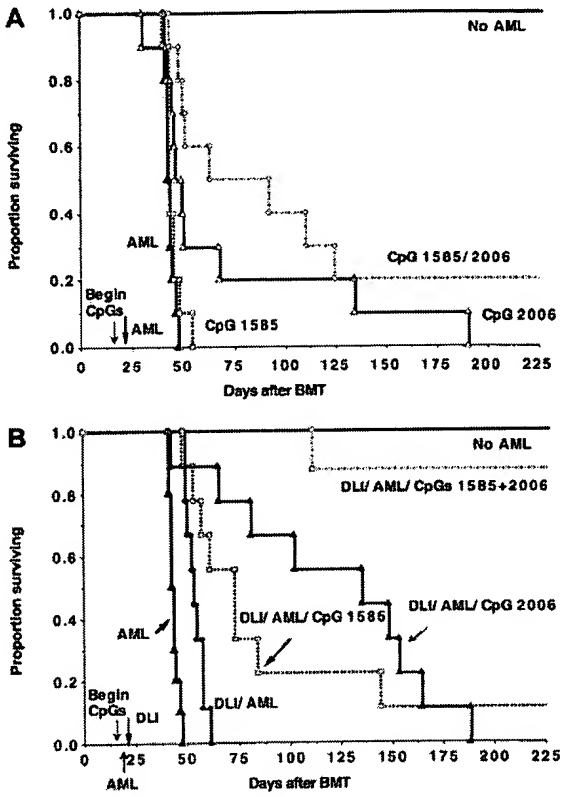

Figure 8. Combined treatment with CpG 1585 + 2006 and DLI markedly prolongs the survival of recipients of allogeneic TCD bone marrow challenged with AML cells early (day 17) after BMT. Lethally irradiated B6 recipients were reconstituted with B10.BR TCD bone marrow (n = 8-10 per group). On day 17, recipients were given C1498 cells (2×10^5). An additional control group received no C1498 cells. Cohorts of mice received no treatment, CpG 1585, CpG 2006, or both ODNs. ODNs treatment were initiated on day 15 and continued through day 43 (A). Other cohorts received DLI (B10.BR splenocytes, 25×10^6/mouse) on day 21 alone or with CpG ODNs (B). (A) As compared with controls, CpG 1585 treatment did not significantly prolong survival ($P = .08$), whereas CpG 2006 alone or CpG 1585 + 2006 significantly prolonged survival ($P = .015$ and .001, respectively). (B) As compared with controls, all groups receiving DLI had a significantly prolongation in survival ($P \leq .0006$). The inclusion of CpG 1585, 2006, or both ODNs along with DLI resulted in an equivalent or in most cases a significant increase in survival as compared with either DLI alone ($P = .01$, .004, and .0001, respectively) or the comparable CpG ODN groups alone ($P = .001$, .10, or .002, respectively).

which was significantly ($P = .001$) superior to controls but ultimately statistically similar ($P = .07$) to CpG 2006 alone. Two (22%) of 9 mice that were autopsied had no evidence of tumor.

As compared with nontreated controls, DLI administered 4 days after C1498 challenge extended survival ($P = .0006$). However, all died within 6.5 weeks after AML cell injection. DLI was similarly effective as CpG 2006 administration at this period after BMT ($P > .50$) (Figures 8A versus 8B). Mean weight curves in DLI-treated groups were modestly but not significantly lower ($P = .08$) than the comparable non–DLI-treated groups (weight curves are not shown). When DLI was combined with CpG 1585, survival was extended greater than with CpG 1585 ($P = .001$) or DLI ($P = .01$) alone (Figure 8B), and 4 (44%) of 9 mice died without evidence of tumor in contrast to the uniform detection of tumor in the other 2 groups. Mean weight curves in the DLI and DLI + CpG 1585 groups were comparable ($P = .96$). When DLI was combined with CpG 2006, survival was modestly but not significantly longer than with CpG 2006 alone ($P = .10$) and was superior to DLI alone ($P = .004$). Moreover, 5 (63%) of 8 mice that were autopsied had no gross evidence of tumor. DLI-induced GVH was enhanced by the inclusion of CpG 2006 with DLI as indicated by the clinical appearance (ruffled fur, hunched posture, cachexia) and lower mean weight curves in DLI + CpG 2006 with or without CpG 1585 as compared with DLI ($P \leq .01$) or CpG ODNs alone ($P < .001$). In contrast to 10% or less survival rate in recipients given DLI and either CpG ODN, the combination of DLI and both CpG ODNs resulted in 88% long-term survival ($P = .0001$ versus DLI; $P = .002$ versus CpG 1585 + 2006) without gross evidence of tumor at necropsy. Thus, the combined administration of DLI and CpG ODN is a highly potent strategy to stimulate anti-AML immune responses in alloBMT recipients of TCD bone marrow grafts at time periods prior to complete immune reconstitution.

Discussion

Several major findings can be derived from our study. First, CpG 2006 is a potent inducer of anti-AML responses in naive as well as syngeneic BMT recipients. In the setting of alloBMT, CpG 2006 also induces an anti-AML response that is bolstered by the inclusion of CpG 1585 and especially by combining both CpG ODNs with DLI. DLI-induced GVHD responses were increased by CpG 2006 administration. Thus, CpG ODNs are potent antileukemia agents that are effective in naive and BMT recipients with minimal residual disease even in the setting in which mice do not have a fully functional adaptive immune system.

We have shown that CpG 2006 but not CpG 1585 ODN pretreatment can reduce tumorigenicity by more than 100-fold in naive mice. CpG ODN was more effective when given 2 days before the AML challenge than concurrently with or following the challenge. To date, the published literature indicates that systemic CpG ODN administration alone may delay but not prevent tumor growth.[7,34-40] Because CpG ODNs each have distinct functions on the immune system, extrapolation to studies using CpG ODNs other than the ones used here is not possible. Results with CpG 2006 for the induction of antitumor immune responses have not been reported, although recent unpublished data (Z. Ballas, A.M.K., 2001) indicate that our findings with systemic administration can be observed in other tumor models. Because CpG ODNs in general stimulate the innate immune system, antitumor studies have focused on developing CpG ODNs as adjuvants for tumor-associated antigens or cellular vaccinations. We have shown that systemic CpG ODNs can induce an antilymphoma immune response when combined with fusion proteins consisting of tumor idiotype protein conjugated to either a neoantigen or granulocyte-macrophage colony-stimulating factor or with an antitumor mAb.[7,34,35] Others have shown that systemic CpG ODNs can enhance the survival of mice that receive peptide or protein vaccines followed by a melanoma cell challenge.[38] Local CpG ODN injection in the vicinity of or at the tumor bed site eradicated tumor and/or led to long-term survival.[36,37] In both instances, CpG ODN generated a memory cell response as assessed by rechallenge experiments.

In our experiments, NK cells were required for the anti-AML responses provided by CpG 1585 or 2006 ODNs as demonstrated by mAb depletion studies and the use of T- and B-cell–deficient SCID recipients. NK-cell depletion did not completely eliminate the protective effect of CpG 2006 ODN in either wild-type or SCID recipients, and TCD did not adversely affect the antitumor immune responses of CpG 2006-treated wild-type mice. Our studies

demonstrated a requirement for NK cells in the optimal AML resistance of mice treated with CpG ODN. T cells were not obligatory for the CpG ODN-facilitated tumor resistance. Consistent with these findings, we could not uncover a memory cell response to AML rechallenge in CpG-treated mice resisting initial AML challenge. Because CpG ODNs are known to stimulate the function of monocytes and DCs, the most likely interpretation of these results is that NK cells are required for optimal antitumor responses, and monocyte/DCs participate by either augmenting indirectly[31] or directly[41] stimulating NK function or by monocyte/DC-mediated antitumor cytotoxicity.[42]

CpG ODN 2006 had the greatest antitumor activity in this model, despite being optimized for activation of human rather than murine cells. This unexpected observation points to the relative conservation in the immune recognition of CpG DNA motifs between different species. Although bacterial and synthetic ODN can induce NK cells to produce IFNγ,[43] IFNγ is neither directly cytotoxic nor cytostatic to the growth of this tumor cell line (not shown). A more attractive candidate protein induced by CpG ODNs is IL-12.[12,28,35] In general, CpG ODNs with phosphorothioate-modified backbones are not as efficient at directly inducing NK-cell function but are more efficient in inducing IL-12 than those with nonmodified backbones.[2,31,32] CpG 2006 is known to be a more potent inducer of IL-12 than CpG 1585 in B6 mice at the biological concentrations likely achieved in this study and is more effective in inducing anti-AML resistance than CpG 1585 in our model. Although IL-12 does affect C1498 growth in vitro, C1498 IL-12 transductants are markedly less tumorigenic than control-transduced cells (unpublished data, 2001). We hypothesize that the superiority of 2006 in our tumor models may relate to its ability to induce relatively strong IL-12 production compared with the other CpG ODNs. Future studies will be required to fully elucidate the responsible mechanisms operative in vivo.

Immature DCs treated with CpG ODN and cocultured with irradiated tumor cells have been shown to provide protection against tumor challenge in vaccinated mice.[39] CpG ODNs are known to activate APCs and to enhance the capacity of APCs to stimulate both $CD4^+$ and $CD8^+$ T-cell responses.[35,44] We hypothesize that APC stimulation induced by CpG 2006 contributed to the antitumor effects. Although CpG 1585 is a potent stimulator of NK function, it is possible that CpG 2006 stimulation of APCs is responsible for the generation of NK effectors and activated monocytes/DCs that work in concert to resist AML. Consistent with this hypothesis, preliminary data indicate that NK-depleted flt3L knockout mice that have a defect in DC numbers were more susceptible to AML-induced lethality than NK-depleted wild-type mice (unpublished data).

An important aspect of our studies was the finding that CpG 2006 ODN administered to syngeneic or allo-TCD bone marrow recipients at early time periods after BMT could augment an anti-AML response. As compared with controls, syngeneic recipients with severe T-cell immune deficiency had a significant survival advantage when given CpG 2006 ODN and challenged with AML cells on day 14 after BMT and when rechallenged later after BMT (day 80). AlloBMT recipients treated with CpG 2006 ODN and challenged on day 36 after BMT also had a significant survival advantage and combined CpG 1585 + 2006 ODNs resulted in 20% long-term survival, even though 2 months or more are required for substantial T-cell immune reconstitution in this setting. In preclinical lymphoma studies, anti-idiotypic mAb given along with a single dose of CpG ODN resulted in 70% versus 40% survival when mAb was combined with 3 doses of IL-2.[31] In naive mice, high-dose IL-2 is not particularly effective in inducing AML resistance,[24] in contrast to CpG 2006 ODN. If proven to be well tolerated in humans, CpG ODNs would represent an attractive alternative to a continuous infusion of low-dose IL-2.

A mainstay of the treatment of leukemia relapse after BMT is the use of DLI. However, DLI is far less effective in acute than chronic leukemia. In our preclinical studies, alloBMT recipients of TCD bone marrow challenged with AML cells on day 17 and then treated on day 21 with DLI had an extended survival, although all died with leukemia. The inclusion of CpG 1585 or CpG 2006 ODNs provided a more potent DLI-facilitated GVL effect and the coadministration of both CpG ODNs with DLI resulted in 88% long-term survival. A potential explanation is the possibility that these CpG ODNs stimulate DLI-derived T-cell and NK-cell effectors, possibly in concert with bone marrow–derived effector cells present at this early time period after BMT. Consistent with the potential effects of CpG ODNs on DLI-derived T cells, recipients given DLI + CpG ODNs had a more severe GVHD reaction than either DLI- or CpG ODN-treated controls. Future studies will be required to determine whether CpG ODNs + DLI differentially affect particular GVHD target organs. Combined administration of DLI + CpG ODNs represents a new approach potentially useful in situations in which DLI alone is incompletely effective in tumor cell reduction.

Th1-like responses dominate the rapid response of the innate immune system to foreign antigens. Immunostimulatory bacterial DNA and synthetic CpG ODNs result in Th1 responses.[6] Although Th1 responses are desirable for cancer immunotherapy, proinflammatory Th1 responses could be detrimental early after BMT. Studies are in progress to explore the effects of CpG ODNs on GVHD and graft rejection. Regardless, CpG 2006 ODNs given with DLI are highly effective in treating AML. Although mice had clinical GVHD, the GVHD was not of sufficient severity to cause lethality in most recipients. Combined CpG ODN and DLI could be especially useful in patients that have acute leukemia typically refractory to DLI.

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Appendix D

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Effects of Sequence of Thioated Oligonucleotides on Cultured Human Mammary Epithelial Cells

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ABSTRACT

We have compared the effects of a number of different oligonucleotides on the growth and morphology of normal finite life span and immortally transformed human mammary epithelial cells. The oligonucleotide sequences chosen initially for study were based on that of the NB-1 gene, which encodes a calmodulin-like protein of unknown function. We found that certain thioated oligonucleotides 15–20 residues in length altered the morphology and decreased the growth rate of the normal cells in a concentration-dependent manner. These effects were rapid, occurring within 24–48 h of oligonucleotide addition. The effects, which occurred without an accompanying detectable decrease in the levels of NB-1 mRNA or protein, were most pronounced in the normal epithelial cells, less apparent in the immortalized epithelial cells, and unobserved in normal breast fibroblasts. Identical sequences having mixed phosphodiester and phosphorothioate backbones, or phosphodiester backbones alone, had little or no effect on normal epithelial cell morphology or growth. Two out of seven additional thioated oligonucleotides which were not complementary to NB-1 mRNA, also affected normal epithelial cell morphology and growth when used at similar concentrations (10 μM). Taken together, the observed effects on normal epithelial cells indicate that certain thioated oligonucleotides may have pharmacological consequences that do not depend on strict complementarity of their sequences to known mRNAs.

INTRODUCTION

SYNTHETIC OLIGONUCLEOTIDES are being used increasingly as inhibitors of specific cell functions *in vitro* and may have therapeutic uses *in vivo* (Wickstrom, 1991). The mechanisms by which these oligonucleotides exert their effects are not well known, but they are thought to involve complementary base-pairing between the oligonucleotides and mRNA molecules, ultimately resulting in degradation or inhibition of translation of targeted mRNAs. Phosphorothioate oligonucleotides, in which one of the oxygens in the phosphate backbone is replaced by a sulfur atom, have received increasing use as antisense reagents because of their greater nuclease resistance than oligonucleotides possessing unmodified phosphodiester backbones (Ghosh and Cohen, 1992). In cell-free systems, phosphorothioates have been shown to form stable hybrids

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with both DNA and RNA, although the melting temperatures of such duplexes are slightly lower than those of phosphodiester oligonucleotides. In intact cellular systems, thioated oligonucleotides have been used successfully to inhibit the expression of a variety of specific mRNAs, including HIV *rev* (Matsukura et al., 1989), *BCL2* (Reed et al., 1990), c-*myc* (Watson et al., 1991), and *cdc2* (Lapidot-Lifson et al., 1992), among others. Specific effects of phosphorothioates have also recently been demonstrated *in vivo*, where antisense c-*myb* thioated oligonucleotides could be shown to suppress the migration and growth of smooth muscle cells (Simons et al., 1992).

In the current study, we utilized specifically designed thioated oligonucleotides in an effort to perturb the expression of the NB-1 gene in normal and immortalized human mammary epithelial cells. NB-1 mRNA, which encodes a calmodulin-like protein, is found in high abundance in normal cultured human mammary epithelial cells, but is decreased in abundance or absent in immortally and tumorigenically transformed derivatives of the same cells (Yaswen et al., 1990, 1992). Our original aim in designing the study was to learn more about the function of this gene through targeted disruption of its expression. We have learned, however, that certain thioated oligonucleotides can affect normal human mammary epithelial cell function in the absence of demonstrable effects on the expression of a target protein. Moreover, our results indicate that different cell types (normal *versus* transformed, epithelial *versus* fibroblast) may behave differently when exposed to the same oligonucleotides. These results could have important implications for the use of thioated oligonucleotides in future experimental and clinical applications.

MATERIALS AND METHODS

Oligodeoxynucleotide synthesis

Oligodeoxynucleotides were synthesized by phosphoramidite chemistry using the applied Biosystem 380B DNA synthesizer (Applied Biosystems Inc., Foster City, CA) and phosphorothioate oligomers were synthesized using the sulfurization agent described by Iyer et al. (1990). After synthesis, oligonucleotides were purified by reverse-phase high-pressure liquid chromatography and precipitated by ethanol.

Cell culture

Normal human mammary epithelial and fibroblast cells were derived from reduction mammoplasty tissue and stored frozen as previously described (Stampfer, 1985). Established cell lines, 184A1 and 184B5, were derived from normal specimen 184 after exposure *in vitro* to the chemical carcinogen benzo[*a*]pyrene as described (Stampfer and Bartley, 1985). All the cells, with the exception of the fibroblasts, were routinely grown and subcultured in medium MCDB 170 (Clonetics Corp., La Jolla, CA) supplemented with EGF (Upstate Biotechnology, Lake Placid, NY), bovine pituitary extract (Hammond Cell Technologies, Palo Alto, CA), insulin, hydrocortisone, ethanolamine, phosphoethanolamine, transferrin, and isoproterenol (Sigma, St. Louis, MO) as described (Stampfer, 1985; Hammond et al., 1984). Fibroblasts were grown in Dulbecco's modified Eagle's medium (University of California, San Francisco, Cell Culture Facility) supplemented with 10% fetal bovine serum and insulin (5 μg/ml). Cell cultures were routinely tested for mycoplasma contamination by Hoechst stain analysis.

To determine the effect of various oligonucleotides on growth in mass cultures, cells were subcultured into triplicate wells in 24-well dishes. Lyophilized oligonucleotides were dissolved in sterile phosphate-buffered saline and added as 100 × concentrates to sparse cultures of growing cells. Culture medium was changed every 2–4 days, during which time the cell morphologies were observed visually. When control wells reached confluence, cells in all the wells were trypsinized and counted using a Coulter counter.

Quantitation of NB-1 mRNA and protein

Total RNA was purified from cells by cesium chloride ultracentrifugation of cell lysates in buffered guanidine thiocyanate, then fractionated by electrophoresis in 1.3% agarose/2.2 M formaldehyde slab gels, and capillary blotted onto Zeta-Probe nylon filters (Bio-Rad, Richmond, CA). The filter was baked, hybridized with ^{32}P-labeled NB-1 cDNA, washed, and autoradiographed exactly as described (Yaswen et al., 1990). Immunoblot analysis of total NB-1 protein was performed by scraping cultured cells into cold PBS, lysing the cells in 1% Triton X-100, 1% sodium deoxycholate, and 1 mM EGTA, denaturing the lysate at 95°C for 10 min. The insoluble material was removed and protein concentration was determined in the remaining soluble lysate by the bicinchoninic acid assay (Pierce, Rockford, IL). Equivalent amounts of total protein were electrophoresed in a 12.5–17.5% SDS-PAGE gel and electroblotted to nitrocellulose and the transferred proteins were fixed in 0.2% glutaraldehyde, blocked overnight with 5% dried milk in PBS, incubated with a 1:1000 dilution of affinity-purified anti-NB-1 antiserum, and visualized using horseradish peroxidase-conjugated secondary antiserum with a chemoluminescent substrate (Amersham, Arlington Heights, IL) (Yaswen et al., 1992). Immunoprecipitation of freshly synthesized NB-1 protein was performed by adding 30 µl of [^{35}S]methionine (10 mCi/ml, 1000 Ci/mmole, Amersham) to 1.3 ml of culture media in each 35-mm dish, incubating for 5 h, followed by scraping cells into 1% Triton X-100, 1% sodium deoxycholate, and 1 mM EGTA, denaturation of the lysate at 95°C for 10 min, removal of insoluble protein, and determination of protein concentration. Lysates containing equivalent amounts of total protein were then adjusted to 200 µl with lysis buffer, incubated with preimmune serum for 3 h at 4°C, followed by Protein A–Sepharose for 1 h at 4°C. Supernatant from this incubation was transferred to a fresh tube, where it was incubated with affinity-purified NB-1 antiserum overnight at 4°C, followed by Protein A–Sepharose for 1 h at 4°C. The beads were then washed four times in 1 ml of lysis buffer, resuspended in sample buffer, heated to 95°C for 10 min, and applied to a 12.5–17.5% acrylamide/20% sucrose gradient gel. The labeled proteins in the gel were visualized by fluorography.

RESULTS

To perturb the expression of NB-1 protein in cultured HMEC, several thioated oligonucleotides (P1–P4) were synthesized corresponding to complementary sequences of NB-1 mRNA. Sequences chosen were either complementary to the 5′ translation initiation site or to regions unlikely to be intramolecularly paired as judged by a computer simulation using the FOLD program (Zucker and Stiegler, 1981). An additional criterion for the choice of antisense sequences was the absence of significant similarity to other known gene transcripts, including those encoding authentic calmodulin. Two sense sequences (P5 and P6), complementary to P1 and P2, were employed as controls. In initial experiments, thioated oligonucleotides were added to cultures of growing cells at final concentrations of 3, 10, and 30 µM, and effects on growth and morphology were monitored. Antisense oligonucleotides P1, P2, and to a lesser extent, P4, had concentration-dependent effects on normal HMEC morphology, apparent within 24 h after their addition to the cell growth media. As illustrated in Fig. 1, normal 184 cells subjected to 10 µM or greater concentrations of P1 or P2 aggregated in patches, adopting less refractile appearances. The number of mitotic cells in these cultures was noticeably decreased by 24–48 h, and did not return to normal in the presence of the *S*-oligonucleotides. The observed effects on cell morphology and mitosis could be reversed during the first 48 h by removal of the *S*-oligonucleotides from the media, indicating that the cells remained viable. Cell counts conducted when control cultures had just reached confluence indicated severe growth inhibition in the normal HMEC incubated with P2 (Fig. 2A). In contrast, the appearance of the normal cells was relatively unaffected by exposure to sense *S*-oligonucleotides P5 and P6, as well as to antisense *S*-oligonucleotide P3 (Fig. 1 and data not shown), although the growth rate was somewhat retarded in normal cells exposed to P6 (Fig. 2A).

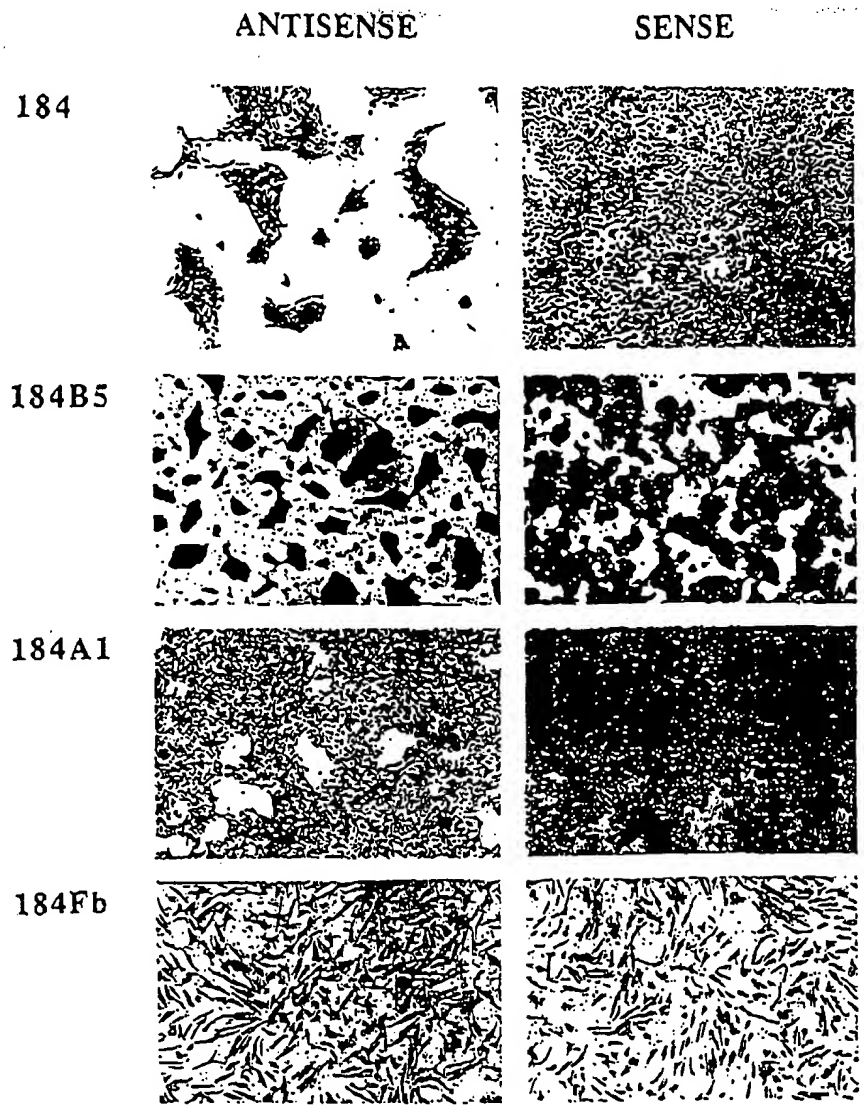

FIG. 1. Effects of thioated antisense oligonucleotides on human mammary cell growth and morphology. Proliferating normal human mammary epithelial cells (184), immortalized human mammary epithelial cells (184B5, 184A1), or normal human breast fibroblasts (184Fb), were exposed to 10 μM concentrations of antisense (P1 or P2) or sense (P5 or P6) thioated oligonucleotides until achievement of confluence or 1 week, whichever came first. In each case, the culture medium was changed every other day. For photomicrography, cultures were washed once in phosphate-buffered saline, fixed in cold methanol, then stained with Giemsa. In the photomicrographs shown, 184 cells were exposed to antisense *S*-oligonucleotide, P2, for 7 days; to sense *S*-oligonucleotide, P6, for 5 days. 184B5 cells received antisense *S*-oligonucleotide, P1, for 5 days; sense *S*-oligonucleotide, P5, for 5 days. 184A1 cells received antisense *S*-oligonucleotide, P1, for 7 days; sense *S*-oligonucleotide, P5, for 7 days. 184Fb fibroblasts received antisense *S*-oligonucleotide, P1, for 7 days; sense *S*-oligonucleotide, P5, for 7 days. Pronounced morphological changes and inhibition of growth were observed in 184 cells exposed to P1 or P2 (both showed equivalent effects), while P5 and P6 showed no such effects. 184B5 showed similar, but less severe response to P1 or P2, whereas 184A1 and 184Fb showed no significant response to these thioated oligonucleotides.

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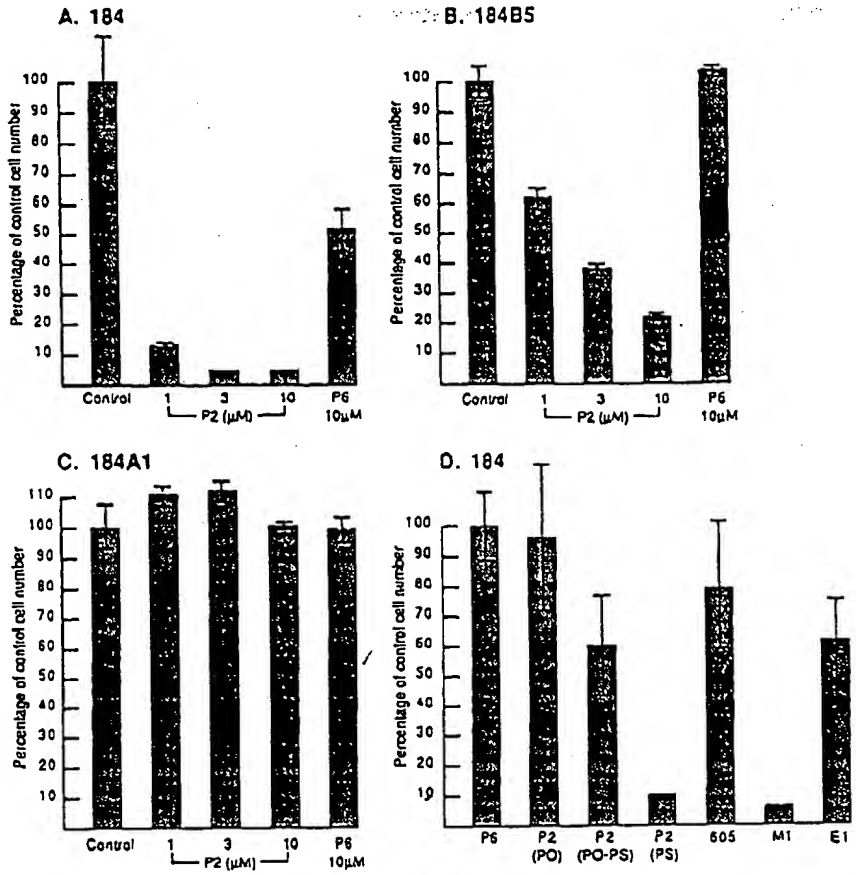

FIG. 2. Quantitation of oligonucleotide effects on normal finite lifespan (A and D), and benzo[a]pyrene-immortalized (B and C) human mammary epithelial cell growth in mass culture. A total of 1×10^4 cells were plated into triplicate wells in 24-well dishes, followed 16–24 h later by exposure to indicated concentrations of oligonucleotides in growth media. The medium with oligonucleotides was changed every 2–4 days until achievement of confluence in control wells, at which time the cells in all wells were trypsinized and counted using a Coulter counter. In A–C, thioated oligonucleotides were used exclusively and the individual bars are mean cell counts ± SEM represented as percentages of mean counts in control wells (no oligonucleotide added). In D, NB-1 antisense (P2) oligonucleotides with all phosphodiester (PO), alternating phosphodiester/phosphorothioate (PO-PS), or all phosphorothioate (PS) buckbones, as well as c-*myc* (M1) and EGF receptor (E1) antisense thioated oligonucleotides were compared with NB-1 sense (P6) thioated oligonucleotides in their relative inhibition of normal (184) cell growth. The concentration of oligonucleotide used was 10 μM in all cases.

S-oligonucleotides P1, P2, P5, and P6 were added to the culture media of immortalized cell lines 184B5 and 184A1, derived from normal 184 HMEC following exposure to benzo[*a*]pyrene, as well as to normal breast fibroblasts. 184B5 cells contain endogenous levels of NB-1 mRNA which are 5- to 10-fold lower than those found in the parental 184 strain, while 184A1 cells and 184Fb fibroblasts do not ordinarily contain detectable levels of this transcript. As shown in Fig. 1, 184B5 exposed to 10 μM P1 or P2 antisense *S*-oligonucleotides showed some morphological aggregation and decrease in growth. However, the effects of *S*-oligonucleotide P2 on growth, as measured by cell counts, was less pronounced in 184B5 than in the 184 parental strain (Fig. 2B). In contrast to 184 and 184B5, 184A1 and the fibroblast strain both showed little or no change in culture morphology in the presence of any of the *S*-oligonucleotides. Effects of P2 on growth of 184A1 or fibroblasts were also minimal in comparison to that seen in 184 HMEC (Figs. 1 and 2C).

To determine whether the observed effects on normal HMEC morphology and growth were accompanied by antisense-mediated inhibition of NB-1 protein expression, cultures exposed to concentrations of *S*-oligonucleotides ranging from 3 to 30 μM for 48 h were extracted with lysis buffer and subjected to immunoblot analysis, using an affinity-purified antibody preparation developed in the laboratory. Surprisingly, steady-state levels of NB-1 protein appeared to be unaltered in 184 cells, even at the highest concentrations of P1 or P2 where obvious effects on morphology and growth of the cultures were noted by 48 h (Fig. 3A and data not shown). Because antisense effects have been shown to be manifested in other systems by destabilization of target mRNAs, we extracted total RNA from 184 cells exposed to P2 or P6 for 4 days, and subjected it to Northern blot analysis. Again, although the cells exposed to P2 showed clear effects of the *S*-oligonucleotide on morphology and growth, no change in the steady-state level of the NB-1 mRNA was observed (Fig. 3B). Finally, we reasoned that prolonged stability of NB-1 protein might mask a decreased rate of synthesis. To measure *de novo* synthesis of NB-1 protein in normal HMEC cultures exposed to the antisense P2 *S*-oligonucleotide or sense P6 *S*-oligonucleotide for 4 days, we labeled the cultures with [^{35}S]methionine for 5 h, before subjecting the exposed cell lysates to NB-1 immunoprecipitation. Again, no difference was noted among antisense P2-treated and control cultures in the amount of newly synthesized NB-1 protein detected (Fig. 3C).

Despite the lack of change in NB-1 mRNA or protein, certain antisense *S*-oligonucleotides were affecting normal HMEC morphology and growth, while other *S*-oligonucleotides were not. We verified the sequence specificity of the phenotypic changes, using different batches of P2 and P6 *S*-oligonucleotides, prepared by ourselves or by a commercial vendor (Operon Technologies, Alameda, CA). Comparison of the *S*-oligonucleotide sequences having a positive effect in our biological assays with those not having such an effect revealed that positive-acting sequences contained a contiguous stretch of at least four dG residues (Table 1). Additional *S*-oligonucleotides were synthesized to test the possibility that the biological effects observed were

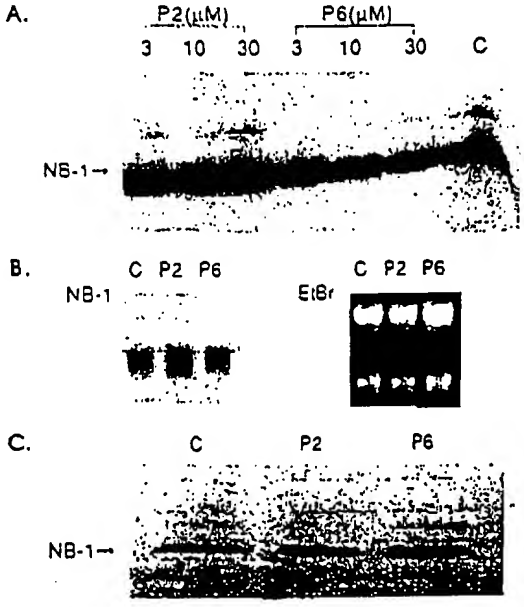

FIG. 3. Comparison of NB-1 mRNA and protein levels in normal human mammary epithelial cells (specimen 184) treated with antisense (P2), sense (P6), or no (C) thioated oligonucleotides. A. Immunoblot analysis of heat-stable cell lysates prepared after 48 h of exposure to indicated concentrations of thioated oligonucleotides P2 or P6. B. Northern blot analysis of total RNA prepared from same cells treated with P2 or P6 for 4 days. The gel was stained with ethidium bromide (EtBr) prior to transfer to show equal loading of total RNA samples. C. Immunoprecipitation of newly synthesized NB-1 protein in 184 cells incubated with P2 or P6 for 4 days.

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TABLE 1. SEQUENCES OF THIOLATED OLIGONUCLEOTIDES TESTED

A. Sequences causing aggregated morphology and cessation of growth in normal human mammary epithelial cells.

P1: 5'- TCT GGG GTG GCA GGT GGA GA -3'

P2: 5'- CAT GCC AGG GGT GGG GGC GT -3'

P4: 5'- CTG GGG CAG CGC GGG AGG CA -3'

Q2: 5'- CTA GCC TGG GGA GGG GGC GA -3'

M1: 5'- AAC GTT GAG GGG CAT -3'.

B. Sequences showing little or no effects on the normal human mammary epithelial cell morphology or growth:

P3: 5'- GGT CGA TCT CAC TCA TCA TG -3'

P5: 5'- TCT CCA CCT GCC ACC CCA GA -3'

P6: 5'- ACG CCC CCA CCC CTG GCA TG -3'

P7: 5'- CAT GCT GCA AGC GCT ACC GG -3'

Q1: 5'- TGG CAG AAG AGG CCA CTG TG -3'

E1: 5'- CGC TGC TCC CCG AAG AGC TC -3'

605: 5'- CCC CGG GGG GGC CCC -3'.

dependent on dG-rich regions rather than strict complementarity to known sequences. Q1, an antisense sequence complementary to the region of NB-1 mRNA slightly upstream of the sequence complementary to P2, but lacking a contiguous stretch of dG residues, had no effect on 184 HMEC morphology or growth (data not shown). In contrast, a nonsense *S*-oligonucleotide. Q2, in which the positions of dA and dT residues were placed opposite their positions in P2, produced the same effects as P2 in 184 HMEC, despite lacking complementarity to NB-1 mRNA (data not shown). Similar results were obtained with antisense *S*-oligonucleotides to other known mRNAs. E1, complementary to the translation start site of the human epidermal growth factor (EGF) receptor mRNA, contained no contiguous stretch of dG residues, and had little effect on 184 HMEC morphology or growth (Fig. 2D). M1, complementary to the translation start site of *myc* mRNA, contained a contiguous stretch of four dG residues, and showed a positive effect identical to that of P2 (Fig. 2D). Although these latter experients suggested a simple correlation between dG-rich *S*-oligonucleotides and the observed effects on normal HMEC, a synthetic *S*-oligonucleotide (605) containing seven dG residues surrounded by eight dC residues was inactive in the biological assays (Fig. 2D). Finally, reasoning that the thioated backbones of the oligonucleotides might influence the specificity of our results, we compared the effects of P2 oligonucleotides with all phosphodiester or alternating phosphodiester/phosphorothioate

backbones to the same sequence bearing an all phosphorothioate backbone. As shown in Fig. 2D, the degree of growth inhibition observed was proportional to the phosphorothioate composition of the sequence.

DISCUSSION

The studies described in this report were originally intended to utilize thioated antisense oligonucleotides to block specifically the expression and thus probe the function of a gene, NB-1, which is expressed in normal HMEC but which is greatly down-regulated or absent in malignantly transformed breast cell lines (Yaswen et al., 1990, 1992). Oligonucleotides bearing phosphorothioate backbones were chosen for this purpose because of their previously documented resistance to nuclease digestion, ability to be taken up by cells, and ability to form stable hybrids with specific RNAs (Ghosh and Cohen, 1992).

When actively growing normal HMEC were exposed to three out of five specifically designed NB-1 antisense *S*-oligonucleotides at concentrations ranging from 3 to 30 μM, characteristic changes in culture morphology and growth rate occurred within 24–48 h of oligonucleotide addition. The affected cells aggregated into patches, became flatter, and ceased cell division. These changes were dependent upon the concentration of the *S*-oligonucleotide used, and could be partially reversed, during the first few days, by removal of the *S*-oligonucleotides from the culture media. Cell types expressing different amounts of "target" mRNA expressed differing susceptibilities to *S*-oligonucleotide administration. However, although the observed biological effects appeared at first to be somewhat sequence dependent, no corresponding decreases in NB-1 mRNA or protein were observed. Furthermore, the ability to exert the observed biological changes appeared to correlate best not with primary sequence of the target mRNA, but with the presence of contiguous stretches of four or more dG residues. The correlation with contiguous dG residues did not hold, however, in one case where dG residues were surrounded solely by dC residues. In retrospect, it is possible that intramolecular base-pairing between the dG residues and dC residues in the latter case may have interfered with a phenotypic effect dependent on four single-stranded dG residues.

In several studies of antisense oligodeoxynucleotides carried out in the past few years, a variety of mechanisms have been delineated (Wickstrom, 1991; Cohen, 1991). In addition to the desired antisense inhibition of expression of the target sequence, which by definition must be sequence-specific and result in decrease of expression of the target mRNA and/or protein (Matsukura et al., 1989; Reed et al., 1990), several non-sequence-specific mechanisms have been described. For phosphorothioate analogs, these include protein binding, both in a nonselective (Ghosh et al., 1992) and in a selective manner (Gao et al., 1989, 1990; Majumdar et al., 1989; Stein et al., 1989), cell-surface binding (Stein et al., 1991a,b) and nonselective translation inhibition (Cazenave et al., 1989; Ghosh et al., 1992), probably due to RNase H cleavage on transient hybridization (Ghosh et al., 1992; Giles and Tidd, 1992). It should be noted that these mechanisms are not restricted to phosphorothioate oligonucleotides, and, in fact, the nonselective RNase H cleavage of mRNA appears to be greater for phosphodiesters than for phosphorothioates at low concentrations (2 μM or less) (Ghosh et al., 1992). However, phosphorothioates also inhibit DNA polymerases and RNase H (Majumdar et al., 1989; Gao et al., 1992). In several applications of phosphorothioate oligodeoxynucleotides in cultured cells, non-sequence-specific mechanisms have been observed, including inhibition of human papillomavirus type 16 (HPV-16) in human cervical carcinoma cells (Storey et al., 1991), and ICAM-1 inhibition in human umbilical and carcinoma cells (Chiang et al., 1991). In other studies, unexpected stimulation of cell growth by certain phosphorothioate oligomers has been observed (Jaroszewski et al., 1990; Tanaka et al., 1992). Such non-sequence- specific effects do not occur uniformly among all cell types. Prior to our studies, Watson et al. (1991) used a thioated anti-c-*myc* oligonucleotide in their studies of the MCF-7 breast tumor cell line. No obvious morphological changes were reported in MCF-7 cells using this oligonucleotide, despite the presence of four contiguous dG residues. Using an identical thioated anti-c-*myc* oligonucleotide in our studies, we observed severe morphological changes in normal human mammary

epithelial strain 184, but little change in the immortally transformed derivative, 184A1. Simons et al. (1992) have also successfully used a thioated oligonucleotide containing four contiguous dG residues for specific antisense inhibition of c-*myb* mRNA in rodent smooth muscle cells.

In the present study, the phenotypic effects occurred fairly rapidly (24–48 h), which is faster than the previously described time for optimal uptake of phosphorothioate oligomers (Matsukura et al. 1988; Loke et al., 1989). Other researchers have indicated faster uptake of phosphorothioate oligomers (Gao et al., 1992), but the usual time required for successful biological assays is of the order of 3–7 days (Matsukura et al., 1989; Reed et al., 1990). Consequently, it is possible that the effects on cell growth and morphology seen here do not result from cellular uptake, but are exerted directly at the cell surface. However, the selectivity of the effects for certain cell types and oligomers indicates a degree of selectivity inconsistent with simple random phosphorothioate binding to the cell surface. To elucidate further the nature of the observed phenomenon, we plan to investigate the fate of ^{35}S-labeled oligomers incubated with both susceptible (184) and unaffected (184A1) human mammary epithelial cells.

The results that we have reported here should serve as a cautionary note for researchers using thioated oligonucleotides and, in general, for studies of oligonucleotides as probes of cellular functions *in vitro* and *in vivo*. Pharmacological activities of these chemicals may be somewhat sequence and cell type dependent, while lacking true antisense specificity. It is important, therefore, that studies relying on antisense effects be properly controlled. This is particularly true for cell biological experiments where the phenotypic assays employed, such as growth assays, are themselves fairly nonspecific, and may be due to a variety of causes.

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